Determination of Sites in the Na^+/H^+ Exchanger Isoform 1 Involved in Cation Translocation

by

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ABSTRACT

Na^+/H^+ exchangers (NHEs) are a family of highly regulated, integral membrane (glyco)phosphoproteins that regulate intracellular pH and cell volume homeostasis. Compounds such as amiloride are known to be competitive antagonists of Na^+ binding and previous structural studies have demonstrated that these antagonists interact with amino acids located in transmembrane domains 4 and 9 (TM4 and TM9). We therefore wished to test the hypothesis that TM4 and TM9 are also part of the cation translocation pore. To this end, cysteine-scanning mutagenesis was used to analyze these regions of the protein. Using a functional cysteine-less NHE1 mutant as a template, cysteine residues were introduced at unique sites in TM4 (between amino acids 166-179), TM9 (342-359) and the putative re-entrant loop (R-loop) between TM9 and TM10 (370-437). These residues were modified using methanethiosulfonate (MTS) derivatives, thiol-specific modifying reagents that will covalently bind to freely accessible cysteine residues. MTS modification of an introduced cysteine residue that faces the aqueous pore should inhibit cation translocation. As expected, these reagents did not inhibit cation exchange through the cysteine-less form of NHE1. MTS-modification of single-cysteine mutants located within TM4 had little effect on cation translocation. By contrast, three mutants located in TM9, H353C, S355C, and G356C, all confer inhibition (45-99%) in the presence of MTSET, a positively-charged MTS derivative. Furthermore, mutant S405C, located in the R-loop/TM10, is inhibited by almost 70% in the presence of MTSET. Comparable results were also obtained with the negatively-charged derivative MTSES, although the inhibition was less pronounced. With the exception of P171C, the kinetic profiles of selected mutants in the absence of the MTS reagents remained similar

to that of the wild type and cysteine-less versions of the exchanger. Our results therefore indicate a role for TM9 and the adjacent R-loop/TM10 in cation translocation.

RÉSUMÉ

Les échangeurs de Na^+/H^+ (NHE) sont une famille de (glyco)phosphoprotéines antiporteurs fortement régulées qui sont impliquées dans le contrôle du pH intracellulaire de même que l'homéostasie du volume cellulaire. Des composés tel que l'amiloride sont reconnus comme compétiteurs antagonistes de la liaison du Na^+. De précédentes études de structure ont démontré que ces antagonistes agissent avec les acides aminés situés aux domaines transmembranaires 4 et 9 (TM4 et TM9). Nous voulions tester l'hypothèse que le TM4 et le TM9 font aussi partis du pore de translocalisation des cations.

A ce jour, nous avons utilisé la technique de mutagénèse par "scan" de cystéines pour analyser ces régions de la protéine. L'introduction de résidus de cystéines à des sites uniques s'est faite à partir d'un mutant fonctionnel de NHE1 sans cystéine aux sites uniques dans le TM4 (entre les acides aminés 166 et 179), TM9 (entre les acides aminés 342-359) de même que dans la boucle P/TM10 (entre les acides aminés 370-437). Ces résidus ont été changés par des derivés de méthanethiosulfonate (MTS), qui sont des réactifs modifiant spécifiquement les groupements thiols en s'attachant de façon covalente aux résidus cystéine libres et accessibles. La modification avec MTS d'un résidu cystéine introduit face au pore aqueux devrait inhiber la translocalisation des cations. Trois mutants localisés dans le TM9: H353C, S355C, et G356C ont tous conféré une inhibition dans des proportions de 45 à 99% en présence de MTSET, un dérivé chargé positivement de MTS. De plus, le mutant S405C situé dans la boucle P/TM10 est inhibé presque à 70% par le MTSET. Par contre, la modification des résidus dans le TM4 n'a eu aucun effet sur la translocalisation des cations. A l'exception de P171C, le profil cinétique des mutants sélectionnés demeure similaire au type sauvage et à la

version sans cystéine de l'echangeur en l'absence de MTSET. Nos résultats indiquent donc un rôle de TM9 et de la boucle P/TM10 dans la translocalisation des cations.

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INTRODUCTION

The electroneutral exchange of intracellular H^+ for extracellular Na^+ is the most efficient means of eliminating excess acid from actively metabolizing cells. This essential physiological process is performed by a family of highly regulated, integral membrane (glyco)phosphoproteins, commonly referred to as Na^+/H^+ exchangers (NHEs). Members of the this protein family have also been implicated in mediating cell volume homeostasis, cytoskeletal organization, cell migration, cellular proliferation and Na^+ (re)absorption across epithelia of the kidney, intestines and other organs. Thus, the NHEs participate in a broad range of fundamental cellular processes (Putney *et al.*, 2002; Orlowski and Grinstein, 1997; Wakabayashi *et al.*, 1997).

Na^+/H^+ Exchanger Gene family

To date, eight NHE isoforms (NHE1-8) have been identified by molecular cloning and partially characterized on the basis of their sensitivity to pharmacological antagonists, their affinity for cations, their cellular and subcellular localization, and their regulation via signal transduction pathways (Orlowski and Grinstein, 1997; Numata and Orlowski, 2001; Goyal *et al.*, 2003). Each isoform is derived from distinct genes that are dispersed throughout the mammalian genome and share ~25-70% amino acid identity, with calculated molecular weights ranging from ~60 to 98 kDa.

NHE1, the first NHE cDNA isolated, was cloned using genetic complementation of exchanger-deficient cells (Sardet *et al.*, 1989). This strategy involved generating a stable, exchanger-deficient murine fibroblast cell line. These cells were transfected with

human genomic DNA and a H^+-suicide or H^+-killing selection process was instituted. Under the conditions of this protocol, Na^+/H^+ exchange activity is selected by the ability of transfected cells to survive NH_4^+-induced intracellular acidification (Pouyssegur, 1985). The transfected human gene coding for the NHE1 was thus cloned by selecting those cell-lines that overexpressed NHE activity. According to the deduced nucleotide sequence, NHE1 is predicted to contain 815 amino acids with an approximate molecular weight of 90 kDa.

Tissue, Cellular, and Subcellular Distribution

NHE1 is found in virtually all mammalian cells and tissues examined to date, and is therefore thought to fulfill "housekeeping" functions. Although the NHE1 isoform is expressed initially in both the apical and basolateral membranes of cultured epithelial cells, following polarization, it becomes restricted mainly to the basolateral membrane (Kuwahara *et al.*, 1994). Furthermore, in the kidney, NHE1 has been shown to localize exclusively to the basolateral membrane of multiple nephron segments (Krapf and Solioz, 1991; Biemesderfer *et al.*, 1992).

NHE2-5 display a more limited pattern of expression. NHE2-4 are found preferentially in the gastrointestinal tract and kidney. NHE2 is largely found on the apical membrane of polarized renal and intestinal cells (Hoogerwerf *et al.*, 1996), although some reports also claim that it can reside basolaterally in certain cell lines (Soleimani *et al.*, 1994). NHE2 mRNA expression is also detected in the adrenal gland, and at much lower levels in skeletal muscle, trachea, kidney, brain, testis, uterus, heart, and lung (Wakabayashi *et al.*, 1997; Wang *et al.*, 1993). Immunological localization

studies have indicated that NHE3 is responsible for Na^+/H^+ exchange across the apical membrane of renal proximal tubule and medullary thick ascending limb as well as intestinal epithelia, and plays a direct role in Na^+ (re)absorption (Hoogerwerf *et al.*, 1996; Bookstein *et* al., 1994a; Biemesderfer *et al.*, 1993). The efflux of protons into the lumen is also important for bicarbonate reabsorption. NHE3 mRNA has also been detected at much lower levels in human testes, ovary, brain, spleen, and placenta (Wakabayashi *et al.*, 1997; Brant *et al.*, 1993). NHE4 mRNA is robustly expressed in stomach, moderately expressed in small intestine and colon, and is present at low levels in kidney, brain, uterus, and skeletal muscle. Since NHE4 is present in the collecting tubule of the renal inner medulla, an environment characterized by high osmolarity, it has been postulated to play a specialized role in volume homeostasis (Bookstein *et al.*, 1994b).

Unlike the other isoforms, NHE5 is found predominantly in non-epithelial tissues. Fully processed transcripts of NHE5 are found primarily in the brain, suggesting that it may play a specialized role in neuronal tissues (Attaphitaya *et al.*, 1999; Klanke *et al.*, 1995). It is speculated that this isoform represents the amiloride-resistant form of the exchanger that is present in hippocampal neurons (Raley-Susman *et al.*, 1991).

Transcripts encoding NHE6, NHE7 and NHE8 are widely expressed in mammalian tissues (Numata *et al.*, 1998; Numata *et al.*, 2001; Goyal *et al.*, 2003). NHE6 mRNA is predominantly expressed in highly metabolic tissues such as brain, skeletal muscle, and heart, but its exact subcellular localization remains highly controversial. Earlier studies suggested that it may be localized to mitochondria, partly accounting for earlier descriptions of monovalent cation/H^+ exchangers in this organelle (Garlid *et al.*, 1995). Others have localized it to membranes derived from the secretory pathway or

recycling endosomes (Brett *et al.*, 2002; Miyazaki *et al.*, 2001; Numata *et al.*, 1998). Recent subcellular fractionation studies in our laboratory have corroborated results obtained in the latter studies, showing preferential localization of NHE6 to endosomal vesicles (*unpublished data*). NHE7 accumulates in the *trans*-Golgi network and associated endosomes. It displays unique functional and pharmacological properties, and is postulated to assist in the maintenance of cation homeostasis of this organelle (Numata *et al.*, 2001). NHE8 constitutes the most recently isolated isoform, and has been suggested to play a role in apical membrane transport in the proximal tubule of the nephron (Goyal *et al.*, 2003). Its functional properties are currently unknown.

Functional Properties

Expression of plasma membrane-type NHE isoforms in plasmalemmal NHE-deficient cells has allowed investigators to characterize their kinetic and functional properties. To date, NHE1-5 have been expressed in chemically mutagenized exchanger-deficient fibroblastic cell lines, such as PS120 cells (Bookstein *et al.*, 1996; Levine *et al.*, 1993; Tse *et al.*, 1993; Wakabayshi *et al.*, 1992), AP-1 cells (Szabo *et al.*, 2000; Kapus *et al.*, 1994; Orlowski, 1993; Yu *et al.*, 1993), and LAP1 cells (Chambrey *et al.*, 1997), in order to empirically ascertain their kinetic, pharmacological, and regulatory properties.

Stoichiometry

Na^+/H^+ exchange via the NHEs is a reversible reaction that is driven solely by the relative transmembrane chemical gradient of Na^+ and H^+ (Demaurex *et al.*, 1997). This process is further typified by a tightly coupled, electroneutral 1:1 (Na^+:H^+) stoichiometry

(Aronson, 1985; Kinsella and Aronson, 1982). Comparisons of NHE-catalyzed net-flux of Na^+ and H^+ across the plasma membrane yielded coupling ratios of 0.6 to 1.2, values that do not differ significantly from the ideal 1:1 ratio of exchange (Aronson, 1985). Furthermore, the intrinsic electroneutral character of the exchange process, with no noticeable alteration in membrane potential upon activation and inhibition of Na^+/H^+ exchange, provides further evidence of a highly conserved 1:1 coupling ratio (Aronson, 1985). An exception to the rule appears to be that of the bacterial Na^+/H^+ exchangers, which exhibit an asymmetric stoichiometry 1:2 (Na^+:H^+) and are therefore electrogenic (Taglicht *et al.*, 1993). In mammalian cells, the Na^+-K^+-ATPase generates an inwardly directed Na^+ gradient that facilitates NHE-catalyzed H^+-extrusion (Aronson, 1985).

Kinetic Profile

The dependence of the rate of exchange on extracellular Na^+ concentration ($[Na^+]_o$) obeys simple, hyperbolic Michaelis-Menten kinetics. The characteristic kinetic profile remains consistent both in native systems (Mahnensmith and Aronson, 1985) and in cell-lines transfected with each particular NHE isoform (Orlowski, 1993; Levine *et al.*, 1997; Yu *et al.*, 1993). NHE4 appears to be an exception to the rule, however, as it displays a sigmoidal dependence on $[Na^+]_o$ when expressed in hypertonically exposed PS120 cells (Bookstein *et al.*, 1996) and a hyperbolic dependence when expressed in DIDS-treated LAP1 cells (Chambrey *et al.*, 1997). The functional significance of such discrepant kinetic properties is not currently understood, but may reflect methodological differences. Empirically determined affinity constants (K_m) for $[Na^+]_o$ range between 3 to 50 mM depending on the isoform (Putney *et al.*, 2002; Szabo *et al.*, 2000; Sastrasinh *et*

al., 1995; Orlowski, 1993; Levine *et al.*, 1993; Yu *et al.*, 1993). The kinetic profile is consistent with the existence of a single Na^+ binding site on the extracellular surface of the protein (Wakabayashi *et al.*, 1997).

Intracellular H^+ binding ($[H^+]_i$), in contrast, exhibits a more complicated kinetic profile. One of the distinctive characteristics of most plasma membrane-type NHEs is their exquisite sensitivity to changes in the intracellular proton concentration. Whereas external Na^+ binding exhibits properties consistent with simple Michaelis-Menten kinetics, H^+ binding to the intracellular face of the protein appears to occur in an allosteric fashion. The empirically determined Hill coefficient for plasma membrane Na^+/H^+ exchange as a function of $[H^+]_i$ is >2, suggesting that multiple intracellular H^+ binding sites synergistically interact to promote rapid extrusion of protons once intracellular pH (pH_i) falls below a particular threshold level (Aronson, 1982). It was therefore proposed by Aronson *et al.* (1982) that a "H^+-modifier site" may exist independently of the H^+-transport site on the intracellular face of the protein and is postulated to be responsible for dictating the distinct "pH activation set-point" of each isoform (Szabo *et al.*, 2000; Kapus *et al.*, 1994; Orlowski, 1993; Levine *et al.*, 1993; Yu *et al.*, 1993; Wakabayashi *et al.*, 1992). One apparent exception to this rule is NHE5, which shows a simple first-order dependence on the H^+_i concentration when ectopically expressed in fibroblastic cells (Szabó *et al.*, 2000), suggesting that allosteric regulation by H^+_i may be isoform-specific. Thus, it is currently unclear whether H^+ activation of exchange occurs as a result of conformational changes that are induced by direct protonation of ionizable groups in the transmembrane region (Wang *et al.*, 1995), or

whether cell-specific regulators allosterically stimulate NHE activity through pH_i-sensitive mechanisms (Wakabayashi *et al.*, 1994a; Wakabayashi *et al.*, 1992).

Cation Selectivity

In addition to their ability to exchange Na^+ and H^+, the NHEs also show varying affinities for other monovalent cations. External H^+, Li^+, and NH_4^+ can inhibit Na^+ translocation in a competitive fashion and can also be translocated across the membrane, whereas K^+ can competitively inhibit Na^+ flux through NHE1 but does not appear to be transported. Unlike NHE1, K^+ does not competitively inhibit Na^+ influx mediated by NHE2 or NHE3 (Orlowski, 1993; Yu *et al.*, 1993; Aronson, 1985). This could therefore be indicative of inherent structural differences in the cation binding sites of NHE1 and the other isoforms.

NHE affinity for external monovalent cations such as H^+ and Li^+ is actually greater than that determined for external Na^+, with the apparent order of affinity for NHE1-NHE3 being $H^+ >> Li^+ > Na^+ >>> K^+$ (Yu *et al.*, 1993). However, the overall V_{max} for the exchange of external Na^+ for internal H^+ is several-fold higher than that recorded for the equivalent reaction using the other monovalent cations (Aronson, 1985). This intrinsic property of the NHEs ensures that the reaction with external Na^+ is favored. This also appears to suggest that either the translocation process itself or "debinding" at the inner surface of the membrane constitutes the rate-limiting step for the exchange of external cations for internal H^+ (Aronson, 1985).

ATP-Dependence

As aforementioned, NHE activity is regulated through the combined chemical gradient of Na^+ and H^+, and therefore does not directly consume metabolic energy (Kinsella and Aronson, 1980). However, the presence of metabolic inhibitors employed to reduce cellular ATP results in dramatic decreases in Na^+/H^+ exchange activity. This phenomenon has been observed both in native systems (Cassel *et al.*, 1986) and in transfected cell lines (Kapus *et* al., 1994; Levine *et al.*, 1993). While ATP-depletion has been demonstrated to result in decreased NHE affinity for internal H^+ (Wakabayashi *et al.*, 1992; Cassel *et al.*, 1986), a number of other studies have also noted a corresponding decrease in V_{max} (Goss *et al.*, 1994; Kapus *et al.*, 1994; Levine *et al.*, 1993). These events occur without a detectable decrease in the quantity of plasmalemmal exchangers (Aharonovitz *et al.*, 2000).

Removal of cellular ATP appears to augment the exchanger's dependency upon intracellular proton levels for activity such that, in the absence of ATP, NHE activity at intermediate intracellular pH levels (i.e., between pH 6.5 to 7.2) is largely abolished. Specifically, the transition between the relatively inactive (resting state, neutral pH) and fully active state (~pH 6.0) occurs over approximately 1 pH unit in the presence of normal cellular ATP levels (i.e., 2-3 mM), while the same transition occurs over 0.3 pH units at acidic pH upon ATP depletion (Cassel *et al.*, 1986). It was therefore proposed that at least two classes of regulatory sites, each exhibiting a different affinity for intracellular H^+, must exist within the protein's structural conformation. ATP depletion would presumably disable the high affinity site, ensuring that the protein is unable to function at intermediary levels over the higher pH_i range. ATP depletion would have no

influence over the lower affinity site, however, which would fully activate the exchanger when exposed to higher concentrations of protons (Cassel *et al.*, 1986).

In accordance with its independence from directly consuming metabolic energy, inhibition of NHE activity following metabolic depletion is not associated with any alterations in the phosphorylation state of the protein (Goss *et al.*, 1994). In fact, NHE1 sensitivity towards ATP persists even after virtually all recognized phosphorylation sites have been eliminated through site-directed mutagenesis (Goss *et al.*, 1994; Wakabayashi *et al.*, 1994a). Direct phosphorylation is therefore deemed to be an unlikely means by which ATP regulates NHE function. The indirect effects of ATP on NHE function, through ATP-modulation of NHE regulatory factors, are discussed in the subsequent section on regulation.

Regulation

A distinctive characteristic of the NHEs is their capacity to be regulated by a diverse number of signal transduction pathways. Stimulation of a variety of cell-surface receptors that include receptor tyrosine kinases, G protein-coupled receptors, and integrin receptors initiate signaling pathways that activate a specific set of NHE-regulatory proteins. To date, NHE isoforms have been demonstrated to exhibit sensitivity to tyrosine kinases, agonists of serine/threonine (Ser/Thr) kinases such as protein kinases A (PKA) and C (PKC), and to the Ca^{2+}–binding proteins calmodulin and calcineurin B homologous protein (CHP). In addition, they have also been noted to display a distinct susceptibility to changes in cell volume. Theorized methods of regulation include phosphorylation, binding of regulatory proteins, and conformational changes that alter the affinity of the transmembrane H^+ transport site.

Phosphorylation

Direct phosphorylation of constituent amino acid residues is a common mechanism by which protein function is regulated in response to a variety of external and internal stimuli. Although NHE1 exists as a constitutively phosphorylated glycoprotein, its intrinsic level of phosphorylation can be amplified in response to growth factors (Wakabayashi *et al.*, 1997; Sardet *et al.*, 1990). Inspection of the primary structure of NHE1 reveals a distinct set of potential phosphorylation sites that include consensus sequences recognized by PKA, PKC, calmodulin (CaM), and proline-directed Ser/Thr kinases such as mitogen-activated protein kinases (MAPKs) (Orlowski and Grinstein, 1997). Growth factor-stimulated phosphorylation appears to be restricted to serine residues, as empirical studies have not been able to establish any evidence for the presence of phosphorylated tyrosine residues (Orlowski and Grinstein, 1997; Goss *et al.*, 1994). In particular, Rho-kinase (ROCK), ribosomal S6 kinase ($p90^{RSK}$), and Nck-interacting kinase (NIK) are three Ser/Thr kinases that have been demonstrated to directly phosphorylate NHE1 (Putney *et al.*, 2002; Takahashi *et al.*, 1999).

Although optimal antiport activity following growth factor-stimulation of receptor-mediated pathways appears to require phosphorylation of the NHE protein backbone (Putney *et al.*, 2002), the degree to which direct phosphorylation regulates the NHEs remains a controversial issue. In essence, there are a number of factors that cannot be explained by a phosphorylation-exclusive model of regulation. For instance, deviating responses have been recorded for individual isoforms expressed in different cellular environments. Furthermore, at least partial regulation is retained by NHE1 even after all

potential phosphorylation sites have been eliminated, while some stimuli alter NHE function without any discernable changes to the phosphorylation state of the protein itself (Orlowski and Grinstein, 1997). Hence, it has been proposed that direct phosphorylation of endogenous NHE residues is a requisite event for receptor-mediated, but not receptor-independent, regulation of the antiporter (Putney *et al.*, 2002).

Ca^{2+}-binding Proteins

Two calmodulin-binding domains have been identified within the cytoplasmic domain of NHE1, one high-affinity site (K_d ~ 20 nM) at amino acids 636-656 and one low-affinity site (K_d ~ 350 nM) at amino acids 664-684. The high-affinity calmodulin site has been demonstrated to exert an autoinhibitory effect on cation exchange, a phenomenon that is relieved upon ligand binding (Wakabayashi *et al.*, 1994b). A model has been proposed in which, upon activation, NHE1 undergoes a conformational change that promotes calmodulin-binding via Ca^{2+}-mediated signaling pathways. This interaction ultimately relieves the protein of the autoinhibitory activity of the high-affinity calmodulin-binding domain and exchange can therefore occur. Additional evidence of the autoinhibitory properties of this domain is provided for by deletion studies. Elimination of the calmodulin-binding domain has been demonstrated to render the antiporter constitutively active, a situation that mimics exchange activity when in the presence of elevated $[Ca^{2+}]_i$ (Wakabayashi *et al.*, 1994b).

Regulation of NHE1 by a second Ca^{2+}-binding protein, calcineurin B homolog protein (CHP), has also been established. This regulatory protein interacts with amino acids 515-530 (numbered according to human sequence; ~ residues 520-535 in rat), a

region that is located at the point in which the cytosolic tail emerges from the lipid bilayer (Pang *et al.*, 2001). CHP-binding appears essential for optimal NHE activity as deletion of the CHP-binding domain diminishes Na^+/H^+ exchange to 5-10% of wild-type. It has been proposed that CHP is constitutively associated with the NHEs such that Ca^{2+}-dependent conformational changes in myristoylated CHP are able to regulate antiporter activity (Pang *et al.*, 2001). In addition, this domain has been further implicated in playing a fundamental role in growth factor-stimulated NHE1 activity (Putney *et al.*, 2002).

G-Proteins

Transfection and microinjection experiments have demonstrated that heterotrimeric and small GTP-binding proteins regulate NHE activity. Specifically, G-protein α-subunits $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ have been noted to activate Na^+/H^+ exchange (Lin *et al.*, 1996; Dhanasekaran *et al.*, 1994). Expression of constitutively active $G\alpha_s$ and $G\alpha_i$ have no appreciable effect on antiporter activity, nor does exposure of cells to bacterial toxins that induce the activity of these G-protein subunits (Putney *et al.*, 2002).

Activation of $G\alpha_q$ is mediated through a PKC-dependent pathway. Inhibition of PKC nullifies NHE1 activation via a number of G-protein coupled receptors including vasopressin, α1-adrenergic, and endothelin-1 (Putney *et al.*, 2002; Martin-Requero *et al.*, 1997). RhoA and/or Cdc42 pathways that activate MEKK-1 regulate $G\alpha_{13}$. Expression of mutationally active RhoA, a low molecular weight GTPase, results in augmented NHE activity presumably through activation of the Rho-kinase, ROCK. As aforementioned, this regulatory protein is responsible for directly phosphorylating serine residues in the

cytoplasmic domain of NHE1 (Hooley *et al.*, 1996). NHE regulation via $G\alpha_{12}$ remains controversial. For instance, while it has been demonstrated to inhibit NHE1 in CCL39 fibroblasts, HEK293 cells, and AP-1 cells (Lin *et al.*, 1996), it activates NHE1 in COS-1 cells (Dhanasekaran *et al.*, 1994). Furthermore, although $G\alpha_{12}$ has been established as an inhibitor of NHE1, it concurrently acts as an activator of NHE2 and NHE3 (Lin *et al.*, 1996).

Phospholipids

Consistent with other ion transporters such as K^+ channels and Na^+/Ca^{2+} antiporters, optimal NHE activity is dependent upon the presence of phosphatidylinositol-4,5-bisphosphate (PIP_2). Specifically, NHE1 contains two positively-charged amino acid clusters in the juxtamembrane cytoplasmic C-terminal region that bind PIP_2 *in vitro* (Aharonovitz *et al.*, 2000). Whether PIP_2 binds to NHE1 *in vivo* is unknown. Notwithstanding, substitution of positively-charged residues at either site with alanine significantly reduces Na^+/H^+ exchanger activity, while mutations at both sites are required for complete loss of both *in vitro* PIP_2-binding and transport activity. This suggests that both sites are necessary for optimal activity of the antiporter (Aharonovitz *et al.*, 2000).

Experimental studies have not yet elucidated the mechanisms by which PIP_2 exerts its regulatory actions. Aharonovitz *et al.* (2000) proposed that direct interaction of membrane-associated PIP_2 to the cytoplasmic tail of NHE1 might maintain the protein in a conformation that is optimal for transport activity. Alternatively, PIP_2 may act vicariously through members of the ezrin/radixin/moesin (ERM) family of proteins, and

that they in turn modulate the transport activity of NHE1. Studies have shown that PIP_2 binds to and modulates the activity of the ERMs (Hirao *et al.*, 1996). This hypothesis is supported by the observation ezrin also binds to NHE1 at a site that overlaps the distal PIP_2 binding site (Denker *et al.*, 1998). Thus, PIP_2-modulation may be required for ERM-NHE1 interactions, an association which in itself may be essential for the regulation of NHE1 by the actin cytoskeleton (Denker *et al.*, 1998).

Polyphosphoinositide phosphorylation exists in a dynamic equilibrium *in vivo* and, within this particular class of molecules, PIP_2 constitutes one of the most phosphorylated species. It was therefore proposed that PIP_2 is responsible for conveying the antiporter's indirect sensitivity to ATP. Supporting evidence for this hypothetical model was provided by the observation that metabolic depletion of cellular ATP is accompanied by a reduction in both total and plasmalemmal PIP_2 levels (Aharonovitz *et al.*, 2000). Furthermore, inhibition of NHE activity following ATP-depletion is comparable to that following PIP_2 hydrolysis, and elimination of putative PIP_2-binding sites significantly disrupts the ATP-dependence of NHE exchange (Aharonovitz *et al.*, 2000). Finally, the PIP_2-binding sites have been mapped to the same region of the protein that is responsible for ATP-sensitivity (Ikeda *et al.*, 1997). However, experimental analysis of this conjecture established that PIP_2 is only partially responsible for the ATP sensitivity of the exchanger. Although mutation of the two PIP_2-binding sites results in profoundly reduced sensitivity of NHE1 to intracellular protons, it does not completely negate the ATP-sensitive component of Na^+/H^+ exchange (Aharonovitz *et al.*, 2000).

It has been speculated that the dependence of NHE1 on PIP_2 levels may apply to the other isoforms as well. All isoforms studied to date have demonstrated an acute sensitivity to ATP. Moreover, NHE2-5 also contain motifs similar to the putative PIP_2–binding sites. Thus, the conserved nature of this particular region of the protein indicates its potential role in the function and/or regulation of the NHE family of proteins.

Cellular Actions

In addition to ascertaining its kinetic and regulatory properties, considerable effort is also being devoted to elucidating the specific roles of Na^+/H^+ exchange in cellular processes. Although cell/organelle volume homeostasis and intracellular pH balance are two well-recognized functions of the NHEs, recent observations have alluded to additional roles for these transporters in cellular proliferation, survival, migration, and cytoskeletal organization. These particular areas of research are particularly intriguing and highlight the importance of studying these transporters if one is to fully appreciate their functional contributions to cell, organ, and ultimately, organismal function.

Intracellular pH Balance and Cell Volume Homeostasis

Ionic gradients across the plasmalemmal and organellar membranes rely primarily on two factors: the intrinsic impermeability of the lipid bilayer to ions, and the presence of ion transport proteins, such as carriers, pumps, and channels, that are embedded within the membrane itself. Assuming H^+ translocation is dictated solely by its electrochemical gradient across the plasma membrane, pH_i should equal ~6.2. However, under standard physiological conditions, pH_i is maintained within a neutral range (~7.2 – 7.4).

Maintenance of steady-state intracellular pH is achieved through the actions of a variety of ion transporters that include the NHEs. Slight increases in cytoplasmic acidity generated by cellular metabolism are sufficient to activate the NHEs, which respond in an allosteric fashion by exchanging intracellular H^+ for extracellular Na^+, thereby averting a potential deleterious shift in pH_i (Wakabayashi *et al.*, 1997).

Hyperosmotic stress also modulates the rate of Na^+/H^+ exchange. In response to a hyperosmotic environment, water located within the cytosol will be compelled to cross the plasma membrane thereby inducing cell shrinkage. This, in turn, stimulates the ubiquitously expressed NHE1 isoform as well as other ion transporters, including Cl^-/HCO_3^- exchange and Na^+-K^+-$2Cl^-$ cotransport, resulting in the net uptake of NaCl and reabsorption of water to restore cell volume (Putney *et al.*, 2002). More recent studies have further determined that the hyperosmotic-induced activation of Na^+/H^+ exchange is due to cell shrinkage, rather than osmolarity or ionic strength (Krump *et al.*, 1997). The mechanism behind this response remains undefined. However, it is thought that the induction of the NHE activity in response to changes in cell shrinkage is mediated through dynamic changes in the actin cytoskeleton. NHE1 in particular has been noted to interact with actin-binding ERM proteins (Denker *et al.*, 2000), while ERM-cytoskeletal interactions have been noted to alter NHE1 distribution and NHE1-regulated organization of the cortical actin filaments. Furthermore, cytoskeletal and associated cytoskeletal elements such as F-actin and myosin light chain kinase appear crucial for serum and cell-shrinkage induction of NHE1 activity (Putney *et al.*, 2002; Shrode *et al.*, 1995).

Cytoskeletal Organization and Migration

The association of specific components of the cytoskeleton with plasma membrane proteins is often required for maintaining appropriate cell shape and for coordinating dynamic rearrangements of the actin-based cellular infrastructure. NHE1, in conjunction with ERM proteins, has been proposed to act as an anchor for cytoskeletal proteins by tethering them to the plasma membrane (Denker *et al.*, 2000), although a comprehensive understanding of the functional nature of these interactions has yet to be achieved.

One physiologically relevant feature of this interaction could involve the clustering of NHE1 in dynamic regions of the cell, such as the lamellipodia, where subtle changes in pH or osmotic pressure may influence local molecular events controlling cell motility and structure. NHE1 displays a characteristic clustering pattern at the leading edge of lamellipodia, and has therefore been suggested to play a role in regulating cellular migratory behavior (Denker *et al.*, 2000). This assumption was supported empirically by observations that pharmacological inhibition of NHE1 impaired directed, but not random, neutrophil chemotaxis and chemokinesis in response to the chemoattractant, *N*-formyl-Met-Leu-Phe (Simchowitz and Cragoe Jr., 1986). Cation translocation appears crucial for NHE1-facilitated cellular migration as expression of NHE1/E266I, a translocation deficient mutant that localizes to the leading edge of lamellipodia and associates with the actin cytoskeleton, disrupts polarized fibroblast migration in standard wound-healing assays (Putney *et al.*, 2002; Denker *et al.*, 2000).

ERM-facilitated NHE1-cystoskeletal associations also mediate focal adhesions and cell shape. Focal adhesions - the regions where cells adhere to the extracellular matrix - are important regulators of cell shape, differentiation, and migration. These

distinct foci are typified by an accumulation of specific focal adhesion proteins, such as paxillin, and integral membrane proteins, such as NHE1 (Denker *et al.*, 2000; Tominaga and Barber, 1998). NHE1, in particular, is required for focal adhesion assembly, as paxillin staining in NHE1 deficient cells appears diffuse and inconsistent. Furthermore, this phenomenon occurs independent of exchange activity, as the antiport-deficient mutant E266I is able to restore abundant and organized paxillin staining at focal adhesions (Denker *et al.*, 2000; Tominaga and Barber, 1998). Cell shape is also influenced by NHE1 expression, as PS120 fibroblasts expressing wild type or E266I NHE1 appear pyramidal in shape, whereas NHE1-deficient PS120 cells appear elongated and fusiform (Denker *et al.*, 2000).

Cell Proliferation and Survival

A marked rise in intracellular pH is one of the defining characteristics of early stage mitogenic activity in cells (Kapus *et al.*, 1994). The NHEs, as regulators of intracellular pH, were thus identified as potential mediators of cell proliferation. This conjecture was confirmed when it was demonstrated that, at neutral or acidic pH, lack of Na^+/H^+ exchange severely impedes the capacity of cells to propagate. Cells expressing a wild type NHE isoform display a more robust growth pattern, as they are able to initiate growth over a greater pH range (Pouyssegur *et al.*, 1984).

Although Na^+/H^+ exchange can promote proliferation, it does not appear to be essential for cellular mitogenic activity. Chinese hamster ovary AP-1 cells, a cell-line deficient for plasmalemmal Na^+/H^+ exchange, can continuously propagate in the presence of serum, although the absolute rate of growth is reduced when compared to those cells

expressing NHE1, NHE2, or NHE3 (Kapus *et al.*, 1994). Growth of untransfected AP-1 cells is also markedly reduced in acidic medium (Kapus *et al.*, 1994).

Consistent with their role in cell proliferation, the NHEs are also able to enhance cell survival. Na^+/H^+ antiport directly alleviates intracellular acidification and decreases in cell volume, two events that are associated with cellular apoptosis (Lang *et al.*, 2000; Li and Eastman, 1995). Furthermore, NHE1 inhibition is one of the series of events that comprise the apoptotic process (Lang *et al.*, 2000; Li and Eastman, 1995). Although a link has been established between NHE activity and cell proliferation/survival, the relative contribution of NHE-mediated changes in cell volume, as compared to NHE-mediated changes in intracellular pH, in relation to apoptosis has yet to be adequately defined.

Pharmacological Properties

Na^+ transport proteins, such as the NHEs, are known targets for inhibition by amiloride, a potassium-sparing diuretic compound, and its analogs (reviewed in Orlowski and Grinstein, 1997). In addition to their differing kinetic properties, the NHE isoforms can be distinguished on the basis of their characteristic sensitivity to distinct pharmacological antagonists. A comparison of the different NHE isoforms expressed in heterologous systems has established isoform-specific amiloride affinities that span over 2 orders of magnitude and exhibit the following rank order: NHE1 >> NHE2 > NHE5 > NHE3 (Khadilkar *et al.*, 2001). Although NHE4 has a putatively low affinity for amiloride and its related antagonists, a direct comparison with the other isoforms has proven difficult. Very specific experimental parameters, such as the addition of 4,4'-

diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) to LAP(-) cells expressing NHE4, must be used in order to detect NHE4 activity and, as such, any attempt to perform an accurate comparison of inhibition properties is ultimately precluded (Chambrey *et al.*, 1997; Bookstein *et al.*, 1996).

Non-amiloride antagonists of the NHEs also exist. Cimetidine, clonidine, and harmaline, although chemically unrelated to amiloride, all contain either an imidazoline or guaninidium moiety and therefore bear some structural resemblance to amiloride-based antagonists (Orlowski, 1993). More selective NHE antagonists have been developed in the form of benzoyl guanidinium derivatives, a family of compounds such as HOE642 (cariporide) and HOE694 that display a distinct chemical similarity to amiloride and its analogs (Scholz *et al.*, 1995; Counillon *et al.*, 1993b). These compounds display a rank order of isoform sensitivity that is similar to that described for amiloride and its analogs, but are effective over a larger concentration range (3-4 orders of magnitude) (Khadilkar *et al.*, 2001). Due to their high specificity for the NHEs, and in particular NHE1, these compounds have been employed therapeutically to prevent cardiac ischemia and reperfusion injuries (Khakilkar *et al.*, 2001; Scholz *et al.*, 1995).

Amiloride-induced inhibition of the NHEs can be reduced in the presence of increasing concentrations of extracellular Na^+. The competitive nature of amiloride inhibition suggests that the external Na^+ and amiloride binding sites are either equivalent or in close proximity to each other (Khadilkar *et al.*, 2001; Warnock *et al.*, 1988; Frelin *et al.*, 1986; Mahnensmith and Aronson, 1985; Paris and Pouyssegur, 1983). However, in chloride-free assay conditions, pharmacological inhibitors of the NHEs have been noted to exhibit non-competitive properties, thereby suggesting that the external Na^+ and

inhibitor binding sites are not identical (Warnock *et al.*, 1988). The presence of mixed-type inhibition appears to indicate a scenario in which multiple amiloride binding sites exist within the structural backbone of the protein. A mutually-exclusive binding site for external Na^+ and pharmacological inhibitors such as amiloride may coincide with the cation translocation site, which would ultimately constitute the competitive drug-binding pocket. Additional antagonist binding sites that exist independently of the cation translocation pathway, and of the external Na^+ binding site, could engender the non-competitive drug-binding properties that have been observed (Warnock *et al.*, 1988). Consistent with this notion, site-directed mutagenesis studies have established the presence of residues that contribute to drug-binding without influencing Na^+ affinity (Khadilkar *et al.*, 2001; Wang *et al.*, 1995; Counillon *et al.*, 1993a).

A current focus of NHE research is the attempt to elucidate specific residues that directly interact with pharmacological antagonists. In order to accomplish this objective, a variety of molecular and biochemical approaches have been employed. One of the earliest investigations utilized a selection strategy involving MPA (*N^5-methyl-N^5-*propyamiloride), an NHE inhibitor, which was designed to obtain amiloride-resistant variants of the NHE1 cDNA transfected into Chinese hamster lung fibroblasts. The mutated NHE1 genes were subsequently isolated, and two amiloride-resistant alleles were identified that contained point mutations at Phe^{165} and Leu^{167} (*numbered according to rat NHE1 sequence*) in the putative transmembrane domain 4 (TM4) (Counillon *et al.*, 1993a). Subsequent to this analysis, Phe^{166} and Gly^{178}, also located in TM4, as well as Gly^{152}, Pro^{157}, and Pro^{158}, three residues located in the exomembrane loop between TM3

and TM4, were also deemed to contribute to amiloride sensitivity (Khadilkar *et al.*, 2001; Touret *et al.*, 2001; Counillon *et al.*, 1997).

Chimeric analysis designed to exploit the differing amiloride sensitivities of the high-affinity NHE1 isoform and low-affinity NHE3 isoform has yielded additional results that indicate the importance of TM9 in antagonist recognition and binding. Homologous segments of the *N*-terminal transmembrane domain were transferred between the two isoforms using unique restriction sites, and a segment composed of 66 amino acids that encompassed TM9 (residues 327-392 of rat NHE1) was determined to confer isoform-specific drug sensitivity (Orlowski and Kandasamy, 1996). Subsequently, site-directed mutagenesis analyses identified Glu^{350} and Gly^{356} as two critical residues that play an influential role in amiloride-NHE interactions. Interesting, Gly^{356} appears to have a greater affect on the binding of the more NHE-specific antagonists, such as 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), as compared to amiloride itself (Khadilkar *et al.*, 2001). Although mutation of these sites does not significantly alter Na^+ affinity, it does significantly hinder efficient cation translocation (Khadilkar *et al.*, 2001).

Structure

For the most part, the innate structural elements of the NHE that confer its various functional properties remain largely undefined. Hydropathy plot analysis of the primary structure of the NHEs predicts a similar transmembrane topology for all isoforms (Engelman *et al.*, 1986; Kyte and Doolittle, 1982). According to the Kyte-Doolittle algorithm, the 500 amino acid *N*-terminal region of the protein is comprised of 12 transmembrane α-helical domains, while the *C*-terminus consists of a large, cytoplasmic

tail of approximately 300 amino acids (Wakabayashi *et al.*, 1997). The *C*-terminal tail of NHE1 resides exclusively intracellularly, as exhibited by its inaccessibility to extracellular antibodies, proteases, and impermeant biotin derivatives (Shrode *et al.*, 1998). However, recent immunological analyses of NHE3 showed that epitopes within the *C*-terminal tail of NHE3 can bind to monoclonal antibodies applied externally to intact cells, suggesting that regions of the *C*-terminal tail of this isoform may protrude through the bilayer to face the extracellular surface despite the absence of readily identifiable hydrophobic α-helices capable of spanning the membrane (Biemesderfer *et al.*, 1998).

The cytoplasmic region contains at least four distinct functional domains that are involved in pH_i sensitivity and antiport regulation (Wakabayashi *et al.*, 2000). The secondary structure of the *C*-terminal tail has been characterized using circular dichroism spectroscopy and has been determined to consist of 35% α-helix, 17% β–pleated sheet, and 48% random coil (Gebreselassie *et al.*, 1998). This diverse array of structural orientations, in conjunction with the low amino acid homology of this region between isoforms (~24-56% amino acid identity), is thought to be indicative of the importance of this region in isoform-specific regulation (Putney *et al.*, 2002; Orlowski and Grinstein, 1997).

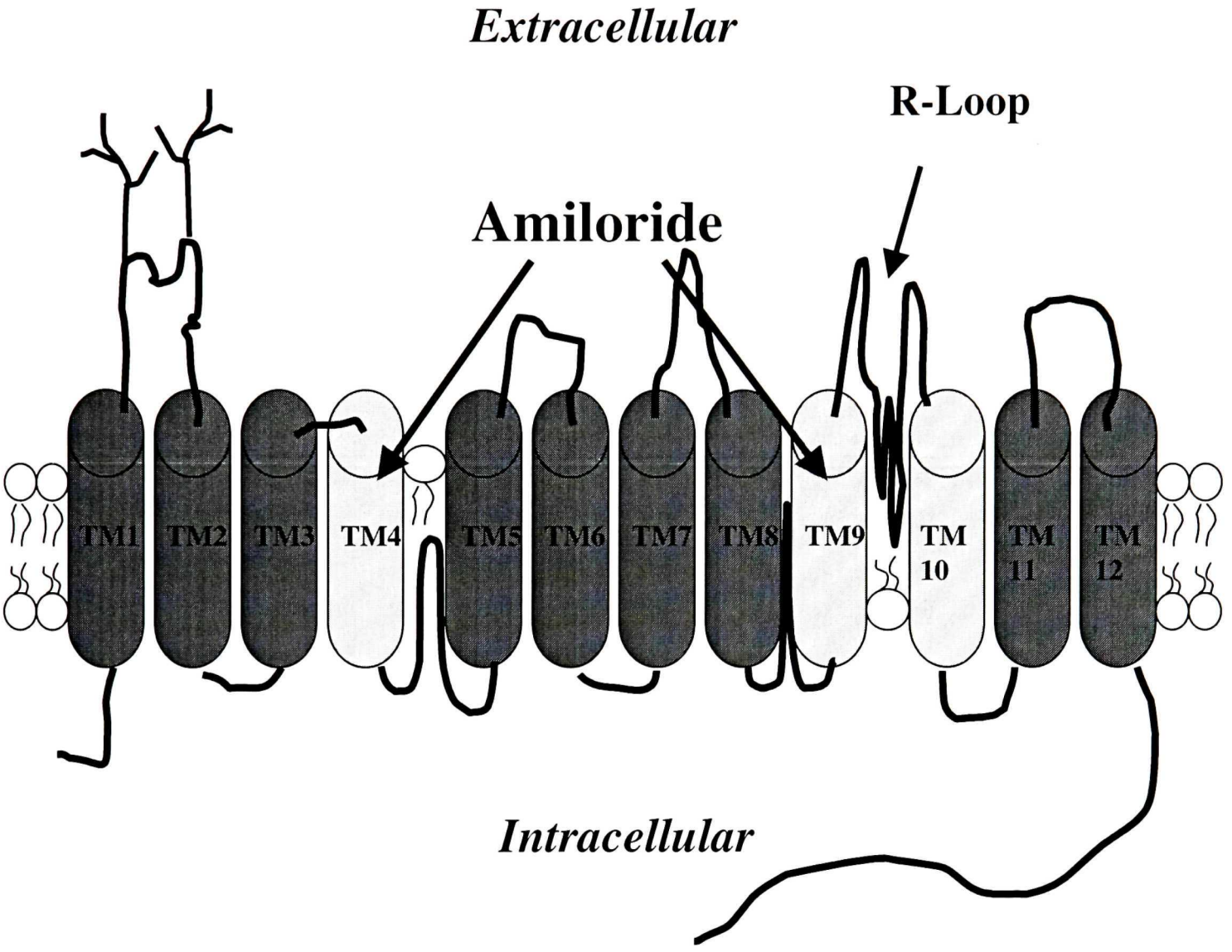
In contrast to the *C*-terminal domain, the *N*-terminus transmembrane domain is highly conserved amongst isoforms (~60% overall amino acid identity between NHE1-5). In addition to the H^+ modifier site, it is thought to contain all the elements directly required for cation translocation (Wakabayashi *et al.*, 1997). Specifically, TM1 and TM2 are the least conserved domains, exhibiting a very limited homology, while TM6 and

TM7 are highly conserved (~95% amino acid identity), therefore indicating a potential role for these two domains in Na^+/H^+ exchange (Orlowski and Grinstein, 1997). It is speculated that the extreme *N*-terminus, including TM1, constitutes a putative signal peptide that is cleaved *in vivo* during protein maturation, although experimental evidence to support this prediction is lacking (Wakabayashi *et al.*, 1992).

Several potential glycosylation sites exist within the primary structure of the NHEs. NHE1 contains both *N*- and *O*-linked glycosylation (Orlowski and Grinstein, 1997). Asn^{75} is a particularly crucial residue as mutation of this amino acid in NHE1 abrogates *N*-linked glycosylation (Counillon *et al.*, 1994). NHE2 exhibits only *O*-linked glycosylation, while no discernable state of glycosylation exists in NHE3 (Tse *et al.*, 1994; Biemesderfer *et al.*, 1993). Although glycosylation has been implicated in proper biosynthetic processing and protein trafficking, its underlying purpose in the NHEs remains elusive.

A recent alternative model to the one predicted by the Kyte-Doolittle algorithm has been proposed by Wakabayashi *et al.* (2000) (Fig. 1). Cysteine-substituted mutagenesis was performed by heterologously expressing single cysteine NHE1 mutants in NHE-deficient PS120 cells. The accessibility of the single cysteine mutants to membrane-impermeant biotin maleimide was assessed in both whole cells and permeabilized cells. Those mutants that were incapable of being covalently labeled in either experimental setting were deemed to contain a cysteine residue that was located in a transmembrane domain. Integration of the resultant data rendered a secondary structural model that, although similar in many respects to the computer-generated hydropathy model, contained a few significant differences (Wakabayashi *et al.*, 2000).

Figure 1. Currently accepted topological model of the *N*-terminal transmembrane domai of NHE1. Note the 12 putative α-helical transmembrane domains and the *N*- and *O*-linked glycosylation sites in the extramembrane loop 1. Amiloride, a potent antagonist of NHE1, interacts with sites located in TM4 and TM9. Amino acid residues in TM4, TM9, the re-entrant loop (referred to as R-loop in the text), and TM10 were identified fo cysteine-scanning mutagenesis.


Extracellular
R-Loop
Amiloride
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
TM9
TM 10
TM 11
TM 12
Intracellular

The prominent discrepancies between the two models concerns the region comprising TM10 and the extracellular loop, EL6. The number of amino acids determined to comprise TM10 and EL6 are not sufficient to span the lipid bilayer twice assuming the transmembrane segments conform to a conventional α-helical configuration. Thus, it was proposed that this region constitutes a putative re-entrant loop (R-loop) that enters the membrane from EL5, invaginates within the lipid bilayer, and then emerges again on the extracellular side before it reenters to form the novel TM10. Analogous structural elements have been implicated in controlling ion fluxes through both ion channels and pumps (Schneider and Scheiner-Bobis, 1997). As a result of this revision, the former intracellular loop 5, IL5, is now surmised to constitute an extracellular loop, while the previous EL6 now forms TM11 (Wakabayashi *et al.*, 2000).

Compared to the primary and secondary structures, very little information has been obtained describing the tertiary and quaternary structures of the NHEs. It has been proposed that they exist in membranes as homodimers that are connected by disulfide bonds in the *N*-terminal transmembrane domain (Fafournoux *et al.*, 1994; Fliegel *et al.*, 1993). However, the contribution of any putative disulfide bonds to homodimerization is unlikely to be significant as cysteine-less versions of NHE1 are fully functional (data presented herein; Wakabayashi *et al.*, 2000). Although definitive empirical evidence has yet to be generated that conclusively proves this putative quaternary interaction, extrapolations may be made from the recent crystallographic visualization of NhaA, the Na^+/H^+ exchanger of *Escherichia coli*. Electron cryo-microscopy was performed using two-dimensional crystals of NhaA, which resolved a structure characterized by 12 tilted, bilayer-spanning helices. The three-dimensional organization of the protein is

demarcated by a linear arrangement of six helices that are situated proximal to a compact bundle of another six helices, with the density for one helix in the bundle not continuous through the membrane (Williams, 2000). The projection map at 4 Å resolution indicates a highly asymmetrical structure in which NhaA monomers interact to form dimers in alternate up-and-down orientations. The six linear helices are theorized to comprise the fundamental dimer-promoting region of the NhaA monomer. Examination of the transmembrane structural orientation of the protein in both the active and inactive conformational states reveals that regions of ion translocation may exist either within the bundle of six helices, or at the interface between the two groups of six helices (Williams, 2000). However, the specific amino acid residues that directly contribute to cation translocation, in either the prokaryotic or eukaryotic NHEs, have not yet been elucidated and this is therefore the focus of my Master's research project.

Substituted Cysteine Accessibility Method (SCAM)

In order to elucidate specific aspects of secondary, tertiary, and quaternary structure, including delineating sites involved in ion translocation, substituted cysteine scanning accessibility method (SCAM) has proven to be particularly effective. A versatile approach that combines genetic and chemical processes, SCAM is designed to facilitate determination of topological features of membrane proteins by taking advantage of the unique reactivity of the cysteine residue. It can be used to resolve a number of distinct structural elements, including identification of residues that line aqueous pores and channels, determination of the diameter of a translocation pathway, and assessment of the structural disposition of the pathway when the protein is constrained in different

conformational states (Karlin and Akabas, 1998). In addition, the location of gates and selectivity filters may be revealed using this technique, while the electrostatic potential profile of ion transporters may be accurately mapped (Karlin and Akabas, 1998).

The SCAM protocol functions on the basis of a number of inherent assumptions. In membrane embedded proteins, an endogenous or engineered cysteine located in the transmembrane domain should be accessible to one of three environments; the lipid-accessible surface, the water-accessible surface, or the protein interior. The channel lining should compose the only water-accessible surface of the transmembrane region. Electrophilic and hydrophilic reagents should react faster with ionized sulfhydryl groups located in the water-accessible surface than with non-ionized residues located in the lipid-accessible surface or protein interior. Finally, modification of cysteine residues that constitute the ion translocation surface should result in a readily detectable and irreversible alteration in protein function (Karlin and Akabas, 1998).

The SCAM protocol consists of a number of well-established steps. An initial prerequisite generally involves engineering a cysteine-less mutant in which conservative substitutions are performed by converting endogenous cysteine residues to alanines or serines. One cysteine residue is then reintroduced into this cysteine-less background. Multiple single-cysteine substituted mutants are then systematically generated and expressed in heterologous cells. Should the introduced cysteine residue of a particular mutant face the aqueous pore, introduction of small, charged sulfhydryl-specific reagents to the cellular medium should promote cysteine modification. This covalent interaction between the cysteine residue and sulfhydryl-specific reagent should impair membrane

protein-mediated transmembrane fluxes through either steric blockage of the pore or charge repulsion (Karlin and Akabas, 1998).

In order to properly apply this protocol, one must be aware of its inherent advantages and precautions. An imperative feature of this procedure is the resilience of membrane proteins to cysteine substitution. Cysteine substitutions are generally very well tolerated by proteins; an essential characteristic that ensures that the requisite number of functional mutants necessary for a thorough analysis may be achieved (Karlin and Akabas, 1998). Furthermore, identification of residues involved in ion translocation is facilitated through the ease by which a systematic analysis of the single-cysteine mutant transport activities can be performed. Since only the single-substituted mutants containing a pore-facing cysteine residue should experience a reduction of activity in the presence of sulfhydryl-specific compounds, this protocol presents an efficient and concrete means by which candidate pore-residing residues can be identified.

While identification of positive results is relatively straightforward, prudent interpretation of negative results is crucial. Failure to induce a reduction in single-substituted mutant transport activity following sulfhydryl-specific modification may occur as a consequence of the introduced cysteine residue residing within the lipid bilayer or protein interior. On the other hand, chemical modification of a cysteine residue located within the translocation pathway may not induce any reduction in transport activity (Li *et al.*, 1999; Karlin and Akabas, 1998). Furthermore, in addition to basic accessibility, reactivity with a cysteine residue also depends upon factors such as the acid dissociation constant of the thiol group, steric constraints of the pore, and, for electrostatically charged reagents, the electric field gradient present within the pore itself

(Karlin and Akabas, 1998). Thus, although these potential caveats exist, with prudent and informed interpretation of the data, SCAM has the potential to provide an unrivalled ability to characterize various structural elements intrinsic to membrane transport proteins.

Rationale

Hence, based on the above considerations, SCAM was selected as an instructive approach to elucidate residues of NHE1 that are involved in ion translocation. This technique has been used with a considerable degree of success in previous investigations designed to reveal the topological disposition of membrane receptors, ion channels, and ion exchangers (Poet *et al.*, 2001; Sheng *et al.*, 2001; Li *et* al., 1999; Tang *et al.*, 1999; Fujinaga *et al.*, 1998; Tang *et al.*, 1998; Dunten *et al.*, 1993; Xu and Akabas, 1993; Akabas *et al.*, 1992). Specifically, this technique has been used to generate structural models of the pore/channel region itself, and has been instrumental in locating sites of antagonist binding, selectivity filters, and channel gates (Poet *et al.*, 2001; Sheng *et al.*, 2001; Tang *et al.*, 1999).

Although ion translocation must intuitively occur through the *N*-terminal transmembrane domain, randomly performing cysteine-scanning mutagenesis would be an onerous task, as this constitutes a substantial region of the protein (~500 amino acids) (Wakabayashi *et al.*, 1997). Therefore, in order to target likely regions involved in ion translocation, previously accrued NHE1 kinetic and pharmacological data were examined. As mentioned above, amiloride and its analogs display both competitive and non-competitive (mixed-type) inhibition of NHE1. In addition, amiloride and its

analogues have been shown to interact with specific sites within TM4 (Phe^{165}, Phe^{166}, Leu^{167}, and Gly^{178}) and TM9 (Glu^{350}, His^{353} and Gly^{356}) and, according to the definition of competitive inhibition, these regions of drug sensitivity may also constitute, or closely associate with, regions of ion translocation. This hypothesis is further substantiated by data derived from second-site revertant mutants of NHE1 which demonstrated that the region of TM4 surrounding P171 and P172 is a structurally important area that is crucial for sodium binding (Touret *et al.*, 2001). Topological analysis of the NHE1 *N*-terminal transmembrane domain has also implicated the membrane-invaginated segments of NHE1 located between TM4-5 and TM9-10 as hydrophilic regions that may form part of the cation translocation pore (Wakabayashi *et al.*, 2000). We thus decided to focus on these areas of NHE1 in order to elucidate endogenous amino acid residues that contribute to cation translocation.

A fully-functional cysteine-less NHE1 mutant ($NHE1_{HA}\Delta C$) was therefore constructed by converting all eight endogenous cysteine residues to serines, and TM4, TM9, and the R-loop/TM10 were systematically targeted for single-cysteine substitutions. Chemical modification of these residues was conducted using MTS-ethylsulfonate (MTSES), and MTS-ethyltrimethylammonium (MTSET). MTSES is negatively-charged at neutral, physiological pH, while MTSET is positively charged (Karlin and Akabas, 1998). Both reagents are approximately the same size, so any discrepancies in their ability to inhibit transport activity can be attributed to their differential charge.

By delineating residues involved in ion translocation, additional insights may be made into potential molecular mechanisms of ion translocation by the exchanger-class of

ion transporters. It should aid in identifying those amino acids that display a functional significance in terms of Na^+ or H^+ binding sites, as well as those residues that maintain the structural integrity of the protein. In conjunction with other studies, it should also provide insight into the conformational architecture of the putative ion translocation pore, suggest possible conformational changes necessary to mediate ion translocation, and indicate potential mechanisms by which the regulatory *C*-terminal cytoplasmic tail regulates ion exchange through the *N*-terminal transmembrane domain.

EXPERIMENTAL PROCEDURES

Materials – Carrier free $^{22}NaCl$ (radioactivity, 5mCi/ml) was obtained from PerkinElmer Life Sciences (Woodbridge, ON). Amiloride, ouabain, and nigericin were purchased from Sigma-Aldrich (Oakville, ON). α-Minimal Essential Medium, fetal bovine serum, G418®, and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Murine monoclonal α-HA antibody was purchased from BabCo (Richmond, CA) while horseradish peroxidase-conjugated goat α-mouse IgG was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). MTSES and MTSET were purchased from Toronto Research Chemicals (North York, ON). All other chemicals and reagents used in these experiments were purchased from British Drug House Inc. (St. Laurent, Quebec) or Fisher Scientific and were of the highest grade available.

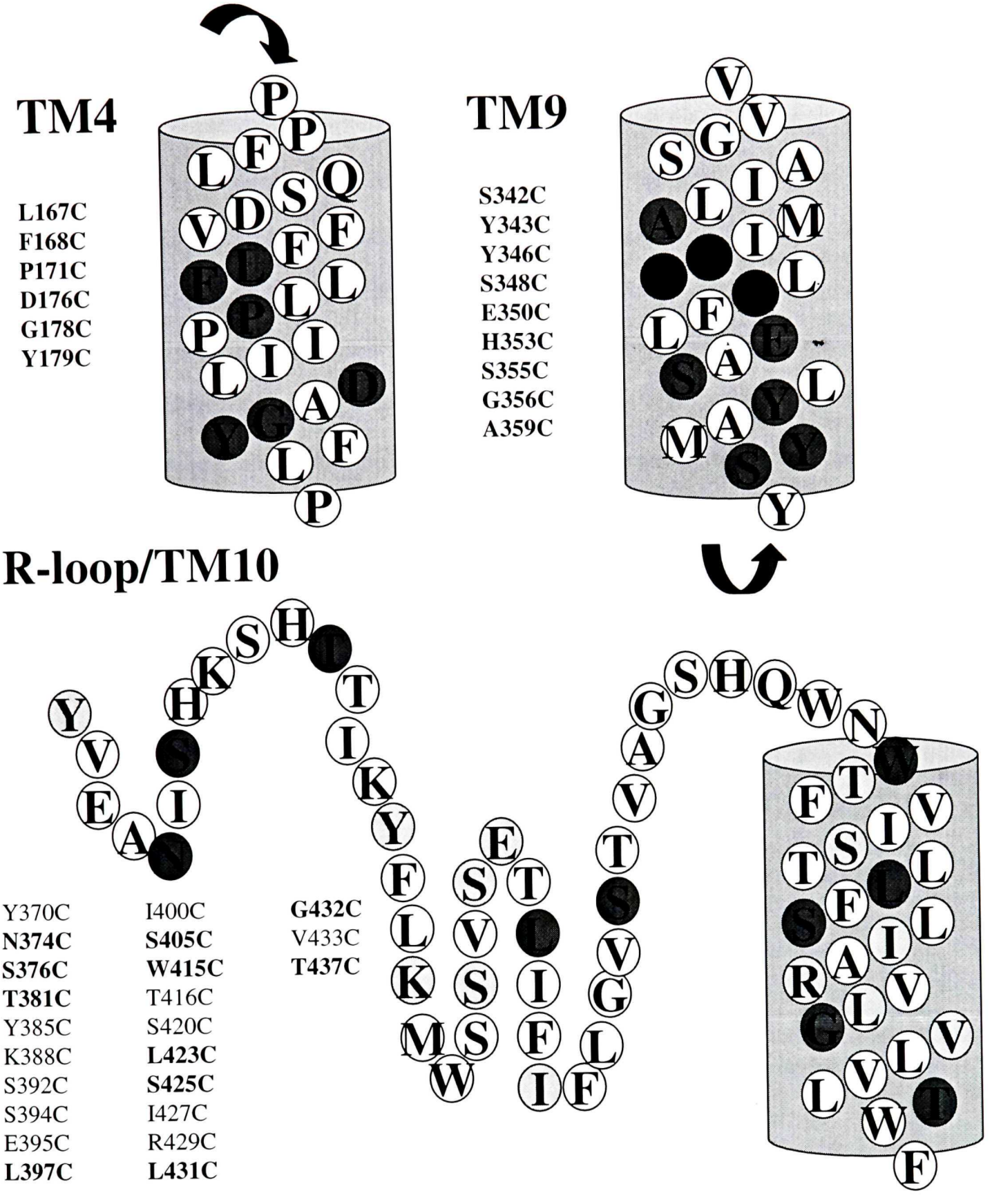
Construction of Na^+/H^+ Exchanger Mutants – The rat cDNA, engineered to contain a series of unique restriction endonuclease sites that provide convenient DNA

cassettes for mutagenesis, was subcloned into a mammalian expression vector under the control of the enhancer/promoter region from the immediate early gene of human cytomegalovirus (pCMV). A single copy of an influenza virus hemagglutinin (HA) peptide (YPYDVPDYA) was affixed to the *C*-terminal amino acid of NHE1 using polymerase chain reaction (PCR) mutagenesis (the construct is referred to herein as $NHE1_{HA}$). $NHE1_{HA}\Delta C$ was constructed by site directed mutagenesis of the eight endogenous cysteine residues (amino acids 117, 137, 216, 425, 481, 542, 565, 799). Conservative mutations were made by converting each cysteine codon to that of serine, according to the mutagenesis protocol devised by Deng and Nickoloff (1992).

Single cysteine residues were introduced into this cysteine-less background using the QuikChange™ Site-Directed Mutagenesis system purchased from Stratagene (La Jolla, CA, USA). Residues in TM4, TM9, and the R-loop/TM10 were selected primarily on the basis of their degree of conservation amongst the isoforms, although other factors such as the charge of the residue and its relative position on a helical wheel model were also taken into consideration (Fig. 2). Mutants were verified using DNA sequencing according to the Sanger protocol supplied with the T7 DNA Sequencing kit (Pharmacia Biotech).

Stable Transfection and Expression of the Na^+/H^+ Exchanger cDNAs – Chinese hamster ovary cells (AP-1 cells), a chemically mutagenized cell-line devoid of endogenous NHE activity (Rotin and Grinstein, 1989), were transfected with a plasmid containing either a $NHE1_{HA}$ or $NHE1_{HA}\Delta C$-based construct using the calcium-phosphate-DNA co-precipitation technique of Chen and Okayama (1987). Cells were maintained in

Figure 2. A schematic representation of the sites in TM4 (Leu^{167} to Tyr^{179}), TM9 (Ser^{342} to Ala^{359}), and the R-loop/TM10 (Tyr^{370} to Thr^{437}) identified for single-cysteine substituted mutagenesis. The arrows are directed from the *N*-terminus of the protein to the *C*-terminus. Completed mutants that have been assayed are represented in bold.


TM4
L167C
F168C
P171C
D176C
G178C
Y179C
TM9
S342C
Y343C
Y346C
S348C
E350C
H353C
S355C
G356C
A359C
R-loop/TM10
Y370C
N374C
S376C
T381C
Y385C
K388C
S392C
S394C
E395C
L397C
I400C
S405C
W415C
T416C
S420C
L423C
S425C
I427C
R429C
L431C
G432C
V433C
T437C

complete α-minimal essential media supplemented with 10% fetal bovine serum and 25 mM $NaHCO_3$ (pH 7.4) and incubated at 37 °C and 5% CO_2.

The selection regimen for producing stable AP-1 cell lines began 48-72 hours following transfection and consisted of a repeated (6-7 times over a 2-week period) acute NH_4Cl-induced acid load (*i.e.* H^+-killing technique) (Orlowski, 1993; Franchi *et al.*, 1986). Positive clonal cell lines were selected and used in the subsequent analysis. To select for those mutants that were unable to survive acid selection, the mutant NHE1 cDNAs were subcloned into the pcDNA3 vectors which co-expresses the aminoglycoside phosphotransferase gene (commonly referred to as the neomycin-resistance gene) and colonies that conferred resistance to the antibiotic G418® (600 μg/mL), an analogue of neomycin, over a two-week period were selected.

$^{22}Na^+$ Influx Measurements – Clonal cells were grown to confluence in 24-well plates and then preloaded with H^+ using the NH_4Cl technique (Franchi *et al.*, 1986; Boron and De Weer, 1976). Initial rates of $^{22}Na^+$ influx were measured essentially as described (Orlowski, 1993). Cells were washed twice and then incubated with an isotonic NH_4Cl solution (50 mM NH_4Cl, 70 mM choline chloride, 5mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 20 mM HEPES-Tris pH 7.4) for 30 minutes in a CO_2-free incubator at 37 °C. Following incubation, the monolayer of cells was rapidly washed twice with isotonic choline chloride solution (125 mM choline chloride, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 20 mM HEPES-Tris, pH 7.4). $^{22}Na^+$ influx was recorded by incubating the cells in isotonic choline chloride solution containing 1 μCi of carrier-free $^{22}NaCl$/mL. The assay medium was K^+-free and was supplemented with 1 mM

ouabain in order to prevent $^{22}Na^+$ transport by the Na^+-K^+-Cl^- co-transporter and the Na^+-K^+-ATPase. $^{22}Na^+$ influx was terminated through the addition of ice-cold isotonic saline solution (130 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES-NaOH, pH 7.4). The monolayer of cells was rapidly washed three times using this solution and then solubilized in 0.25 mL of 0.5 N NaOH. Solubilized cell extract was then washed with 0.25 mL of 0.5 N HCl and added to scintillation vials. Radioactivity was assayed using a liquid scintillation counter. According to the conditions of H^+-loading used in this study, $^{22}Na^+$ uptake is linear for the first 8-10 minutes (at low Na^+ concentrations and at 22°C). Thus, $^{22}Na^+$ influx was recorded following 5 minutes of exposure to $^{22}Na^+$ except when measuring the kinetics of NHE1 activity as a function of extracellular Na^+ concentration ($[Na^+_o]$).

When assessing the K_m for $[Na^+_o]$, a modified version of the aforementioned protocol was instituted. Previous investigations have demonstrated that when $[Na^+_o]$ is increased to 100-125 mM, $^{22}Na^+$ uptake is linear for up to 4 minutes. Therefore, when conducting this kinetic analysis (in which $[Na^+_o]$ concentration ranges from 1.25 mM to 125 mM), $^{22}Na^+$ uptake was terminated following 1 minute. Protein content was assessed using the Bio-Rad *DC* protein assay kit as per the manufacturer's protocol. Rates were expressed as nmol Na^+/min/mg protein.

To examine NHE activity as a function of intracellular H^+ concentration, pH_i was set over a range of 5.4-7.4 using the K^+-nigericin method as previously described (Aharonovitz *et al.*, 2000). Briefly, cell monolayers were washed twice with isotonic choline chloride solution and then incubated for 4 minutes at 22 °C in N-methy-D-glucamine balanced salt solutions (2 mM NaCl, 1mM $MgCl_2$, and 10 mM HEPES, pH

7.4) containing the Na^+/K^+ exchange ionophore, nigericin (10 μM). These pH_i clamp solutions are designed to fix the pH_i at a desired level by adjusting the extracellular K^+ concentration. In essence, the desired pH_i can be established according to the following equation: $[K^+_i]/[K^+_o] = [H^+_i]/[H^+_o]$, assuming that the intracellular K^+ concentration is 140 mM and the extracellular H^+ concentration is set at 7.4. $^{22}Na^+$ uptake was initiated in the same pH_i clamp solutions supplemented with 1 μCi/mL of $^{22}Na^+$ and 1 mM ouabain in the absence or presence of 2 mM amiloride. Uptake occurred for a period of 10 minutes and was terminated in the same fashion as described above.

Measurements of $^{22}Na^+$ influx specific to NHE1 were determined as the difference between the initial rates of H^+-activated $^{22}Na^+$ influx in the presence and absence of 1-2 mM amiloride (a concentration sufficient to inhibit NHE1 under these experimental conditions) and expressed as amiloride-inhibitable $^{22}Na^+$ influx.

Transport Inhibition Assay – Cell monolayers plated in 24 well plates were incubated in PBS-CM (1 x PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) with or without 1 mM MTSET or 10 mM MTSES at 37°C for 30-35 minutes. Cells were then washed twice with isotonic choline chloride solution and incubated for 4 minutes at 22°C in pH_i 5.6 clamp solution. $^{22}Na^+$ uptake was initiated using pH_i 5.6 clamp solution supplemented with 1 μCi of $^{22}Na^+$ and 1 mM ouabain in the absence or presence of 1 mM amiloride. $^{22}Na^+$ uptake was terminated after 10 minutes using ice-cold isotonic saline solution in the same fashion as aforementioned. Inhibition was determined as the ratio of amiloride-inhibitable $^{22}Na^+$ uptake in the presence of MTSES or MTSET to amiloride-inhibitable $^{22}Na^+$ uptake in the absence of these compounds.

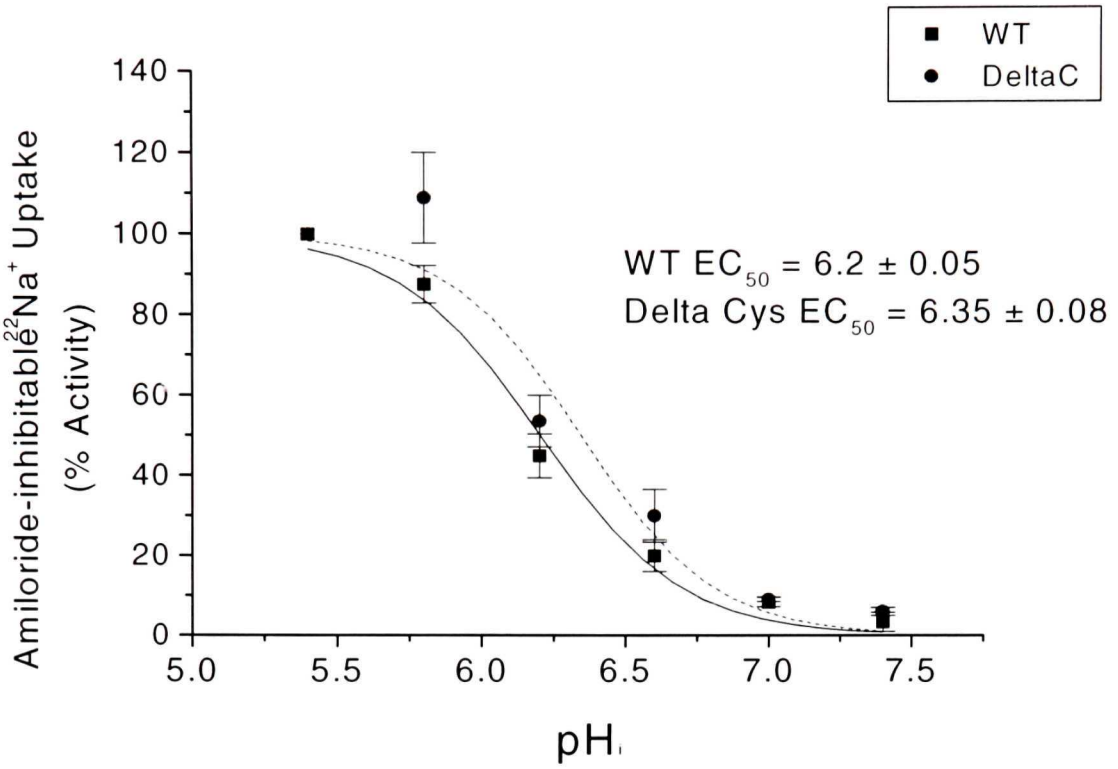
Immunoblotting – Stably transfected cells were grown to confluence in 10 cm plates and were lysed using 1% Triton X-100. Total cellular protein extracts (30 μg) were resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF Hybond-P™ membranes (Amersham Pharmacia Biotech). The blots were rinsed briefly with PBST (1 x PBS containing 0.1% Tween 20), blocked with 5% non-fat skim milk in PBST, and then incubated with a murine monoclonal anti-HA antibody (dilution 1:10 000). Following extensive washing with PBST, blots were incubated with goat anti-murine IgG secondary antibody conjugated with horseradish peroxidase (dilution 1:20 000). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) and recorded on X-ray film.

RESULTS

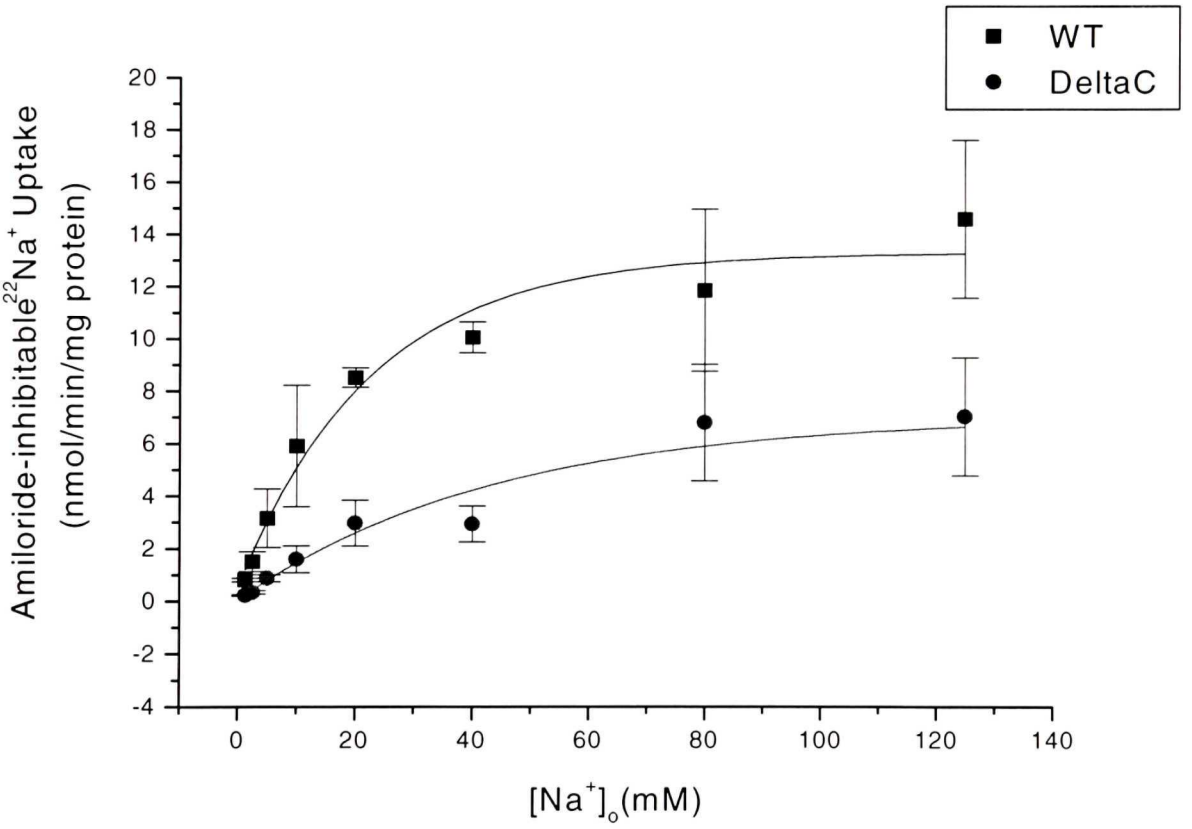
Characterization of $NHE1_{HA}\Delta C$ and the Single-Substituted Cysteine Mutants: $NHE1_{HA}\Delta C$ was constructed according to the abovementioned protocol and expressed in AP-1 cells. A preliminary comparison of the kinetic characteristics of wild-type $NHE1_{HA}$ and $NHE1_{HA}\Delta C$ was performed to ensure that the elimination of the eight endogenous cysteine residues did not significantly perturb protein function. Relative to wild-type, the cysteine-less mutant of NHE1 showed no change in its sensitivity to pharmacological antagonists (*data not shown*) and intracellular H^+ concentration (Fig. 3a), but did exhibit a modest 2-fold reduction in its affinity for external Na^+ (Fig. 3b). Based on these data, it was concluded that the replacement of endogenous cysteines with serines was not

Figure 3. A comparison of the kinetic properties of wild type $NHE1_{HA}$ (WT) and $NHE1_{HA}\Delta C$ (Delta Cys). The empirically derived EC_{50} for intracellular H^+ (A) and K_m for extracellular Na^+ (B) did not differ significantly between constructs. Each data point represents an average of three experimental trials (± S.E.)

A


WT
DeltaC
WT EC50 = 6.2 ± 0.05
Delta Cys EC50 = 6.35 ± 0.08
Amiloride-inhibitable 22Na+ Uptake
(% Activity)
pHi
140
120
100
80
60
40
20
0
5.0
5.5
6.0
6.5
7.0
7.5

B

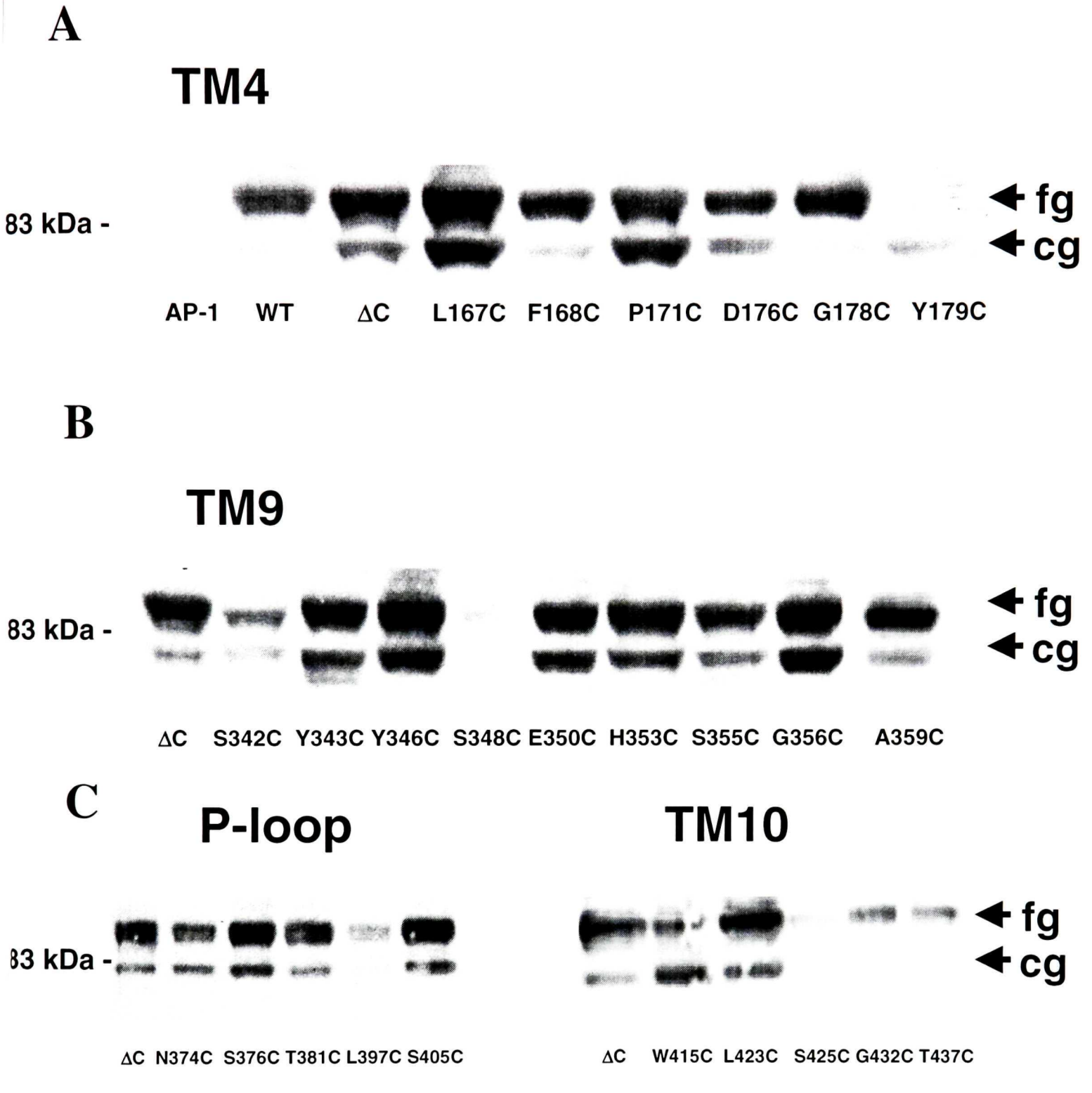

WT
DeltaC
Amiloride-inhibitable 22Na+ Uptake
(nmol/min/mg protein)
[Na+]o(mM)
20
18
16
14
12
10
8
6
4
2
0
-2
-4
0
20
40
60
80
100
120
140

detrimental to NHE1 activity and that $NHE1_{HA}\Delta C$ could serve as a suitable template for further mutational and functional analyses.

To this end, single-cysteine substitutions were generated in TM4 (Leu^{167}-Tyr^{179}), TM9 (Ser^{342}-Ala^{359}), the R-loop/TM10 (Tyr^{370}-Thr^{437}) of $NHE1_{HA}\Delta C$ (Fig. 2), and the resulting constructs were then transfected into AP-1 cells. Usually six clonal isolates (of ~ 15-20 colonies per plate on average) were selected following the rigorous selection protocol described above. The $^{22}Na^+$ influx assay was then performed on selected colonies, and the clonal isolate exhibiting the highest uptake activity for each mutant was subject to subsequent analyses. Cells expressing P171C, S348C, and A359C were of particular interest as only 1-3 clonal isolates were able to survive the selection regime, suggesting that transport activity was compromised. In addition, the Y179C mutant was found to be non-functional.

To make quantitative comparisons of the intrinsic rates of transport of parental and single-cysteine substituted mutants of $NHE1_{HA}\Delta C$ in stably transfected cells, we determined the cellular rates of amiloride-sensitive H^+-activated $^{22}Na^+$ influx and the abundance of fully-glycosylated, cell surface NHE1 protein (the latter assessed by Western blotting). From these values, a normalized NHE1 activity value was calculated as described previously (Khadilkar et al. 2001). Briefly, normalized NHE1 activity was defined as pmol of amiloride-inhibitable Na^+ transported intracellularly *per* minute *per* mg total cellular protein *per* cellular abundance of the fully-glycosylated NHE1. Levels of expression for $NHE1_{HA}$, $NHE1_{HA}\Delta C$, and the single-cysteine substituted mutants are displayed in Fig. 4a, b, and c. The slower migrating band at ~100 kDa represents the completely processed form of the protein that is expressed at the cell surface, whereas the

Figure 4. Western Blot analysis displaying the NHE1 expression levels in untransfected AP-1 cells and cells expressing wild type $NHE1_{HA}$ (WT), $NHE1_{HA}\Delta C$ (ΔC) and the single-cysteine substituted mutants of TM4 (A), TM9 (B), and the R-loop/TM10 (C). The slower migrating band represents the fully glycosylated (fg) form of the protein, while the faster migrating band represents the core glycosylated (cg) form of the protein.


A
TM4
83 kDa -
fg
cg
AP-1
WT
ΔC
L167C
F168C
P171C
D176C
G178C
Y179C
B
TM9
83 kDa -
fg
cg
ΔC
S342C
Y343C
Y346C
S348C
E350C
H353C
S355C
G356C
A359C
C
P-loop
TM10
83 kDa -
fg
cg
ΔC
N374C
S376C
T381C
L397C
S405C
ΔC
W415C
L423C
S425C
G432C
T437C

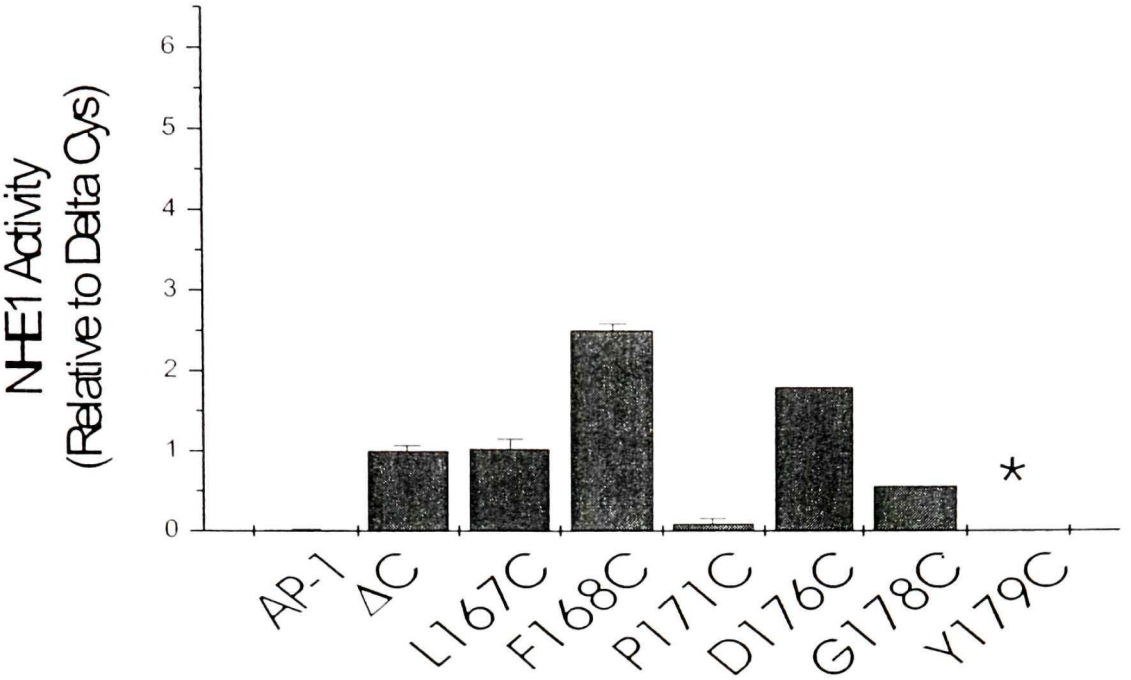
faster migrating band at approximately 75 kDa comprises the partially processed or core-glycosylated form of the protein that is retained in the endoplasmic reticulum (ER) (Shrode *et al.*, 1998). In order to include the non-functional Y179C mutant in the analysis, we subcloned Y179C into the pcDNA3 vector, which co-expresses the neomycin-resistance gene, and selected colonies that survived in the presence of the neomycin analogue G418. As is evident from the Western blot, the modest amount of protein that is expressed appears to be core glycosylated as only the faster migrating band of Y179C is detected (Fig. 4a), suggesting that the protein is retained in the endoplasmic reticulum. In contrast, although the S348C protein is also expressed at low levels, perhaps explaining why only one clonal isolate expressing this protein could be rescued, it is capable of being processed to the fully glycosylated form that resides at the cell surface (Fig. 4b).

In the majority of cases, the activity levels of the plasmalemmal fraction of the single cysteine-substituted mutants did not differ significantly from the parental $NHE1_{HA}\Delta C$ (Fig. 5a, b, c). The notable exceptions were P171C, S348C, S425C, and T437C. The P171C mutation drastically reduced transport activity, as the relative uptake of $^{22}Na^+$ into cells expressing P171C was only marginally greater than untransfected AP-1 cell line (Fig. 5a). On the other hand, the S348C, S425C, and T437C mutations caused a dramatic increase in transport activity. Despite its low expression, the turnover rate for S348C was almost 25 times that of $NHE1_{HA}\Delta C$ (Fig. 5b), while cells expressing S425C and T437C exhibited approximately 10-fold increases in activity (Fig. 5c).

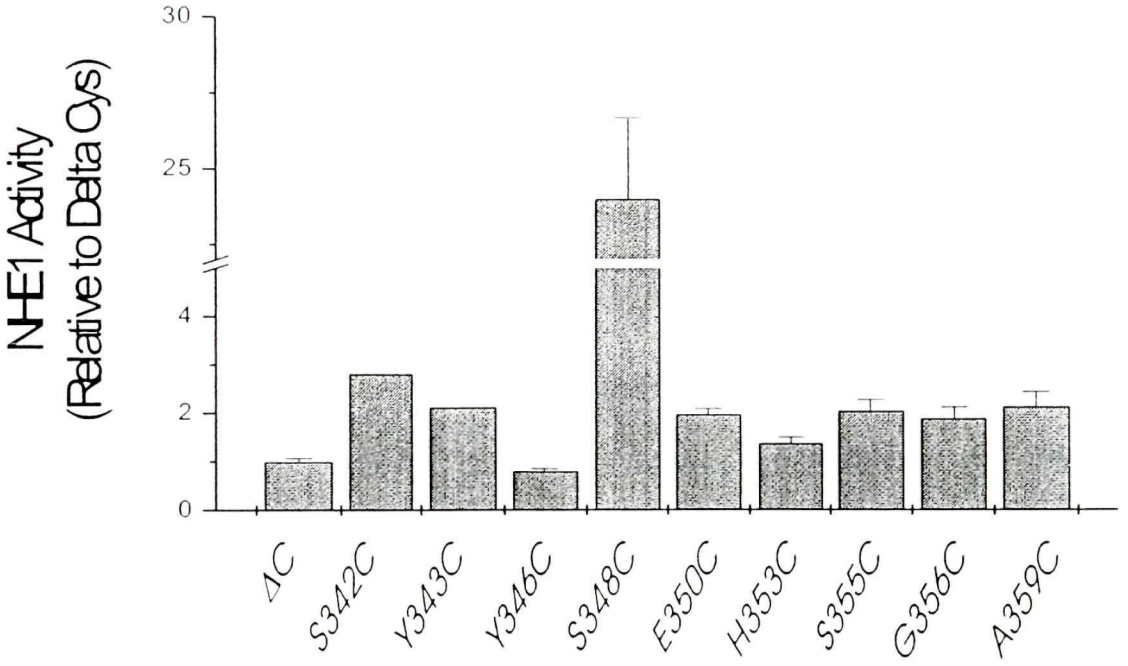
MTS Inhibition of the TM4 and TM9 Single-Cysteine Mutants: Single-cysteine substituted mutants in TM4 and TM9 were analyzed first for their sensitivity to thiol-

Figure 5. Na^+/H^+ exchange activity (pmol Na^+/min/mg protein/IDV relative to $NHE1_{HA}\Delta C$) of untransfected AP-1 cells and cells expressing the single cysteine substituted mutants as a percentage of $NHE1_{HA}\Delta C$'s activity. Single-cysteine mutants of TM4 (A), TM9 (B) and the R-loop/TM10 (C) are displayed. Y179C was non-functional and therefore could not be included in the analysis. Bars represent the average of two experimental trials (± S.E.).

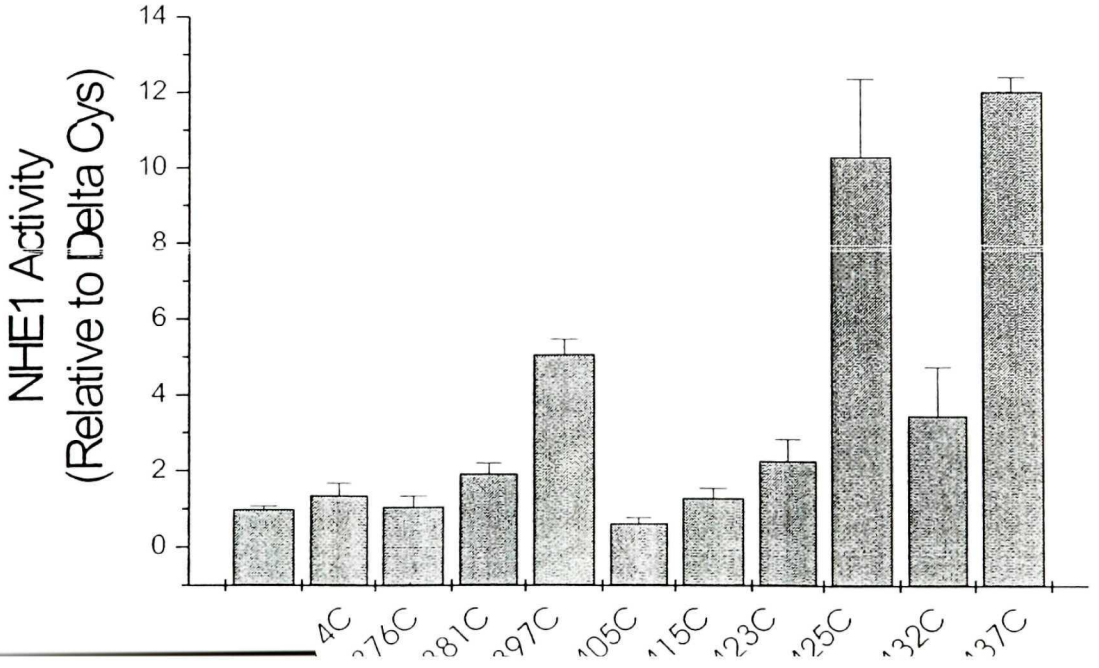
A


NHE1 Activity
(Relative to Delta Cys)
0
1
2
3
4
5
6
*
AP-1
ΔC
L167C
F168C
P171C
D176C
G178C
Y179C

B


NHE1 Activity
(Relative to Delta Cys)
0
2
4
25
30
ΔC
S342C
Y343C
Y346C
S348C
E350C
H353C
S355C
G356C
A359C

C


NHE1 Activity
(Relative to Delta Cys)
0
2
4
6
8
10
12
14

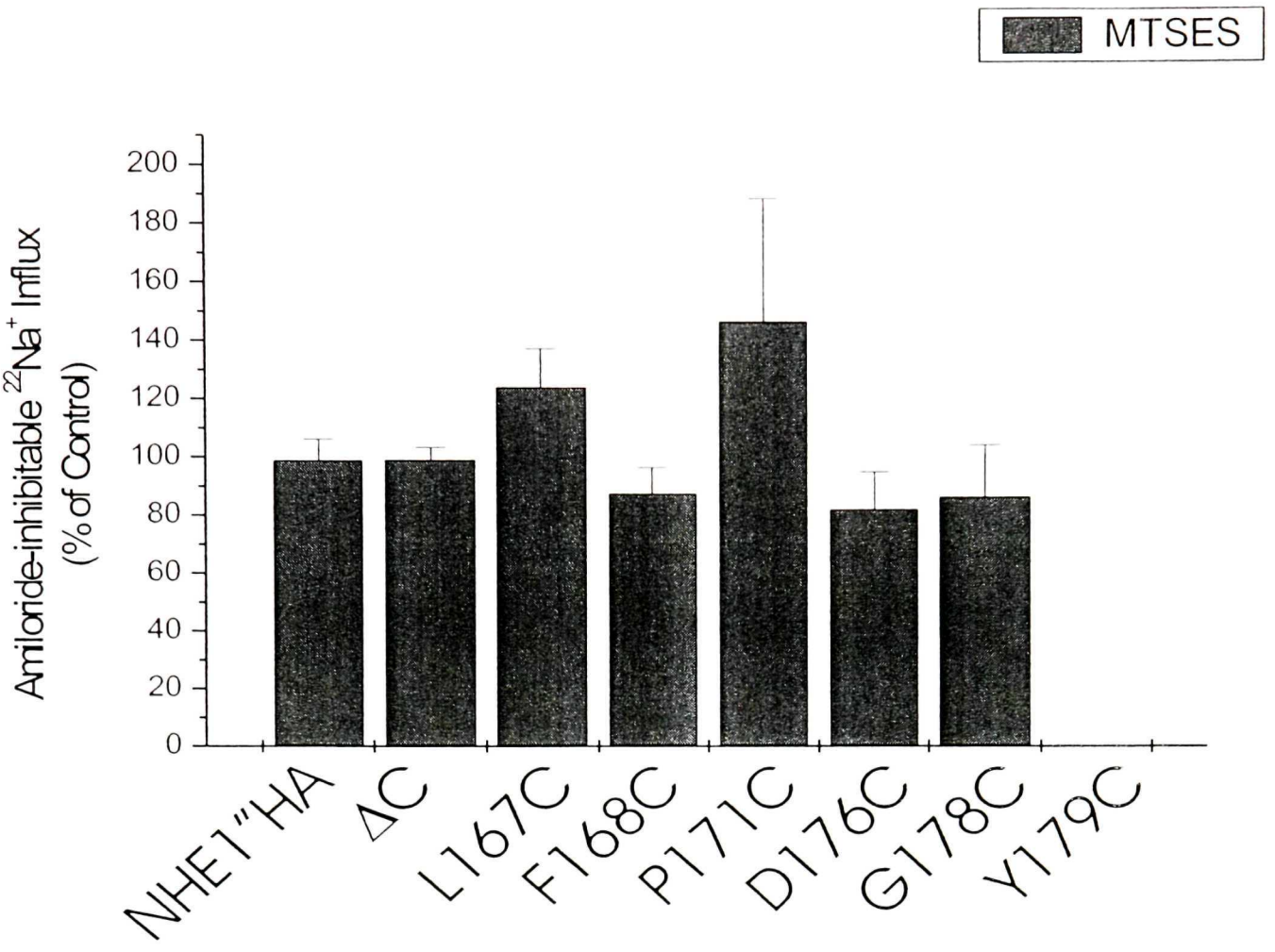
modifying reagents. Cells expressing TM4 single-cysteine substituted mutants experienced little or no reduction in transport activity in the presence of either MTSES or MTSET (Fig. 6a and b). Although the activity of P171C was modestly augmented in the presence of both MTSES and MTSET, it was not statistically significant ($p > 0.05$). As mentioned above, the activity of P171C is very low and difficult to measure accurately, thereby accounting fo: the large standard error.

In contrast to the TM4 mutants, the results clearly indicate a role for TM9 in cation translocation (Fig. 7a and b). Treatment of cells with the positively-charged reagent, MTSET caused a 98.5% reduction in $^{22}Na^+$ transport by the G356C mutant (Fig. 7b). In addition, mutants with cysteine substitutions at neighbouring sites, H353C and S355C, also showed approximately 40% inhibition of activity in the presence of MTSET. Collectively, these data suggest that Gly^{356} and nearby adjacent residues comprise a segment that is critical for ion translocation. Although the extent of inhibition is less pronounced in the presence of MTSES, which is negatively charged, a similar trend is always observed (Fig. 7a). Since both reagents are similar in size, it is likely that the differing charge of the MTS reagents accounts for the differences in their relative levels of inhibition.

Kinetic Characterization of P171C, H353C, S355C, and G356C: Having identified His^{353}, Ser^{355}, and Gly^{356} as residues that likely constitute part of the translocation pathway, we next investigated whether cysteine-substitutions at these sites influenced cation binding in the absence of the MTS reagents. Therefore, kinetic analysis was performed in order to elucidate the extracellular Na^+ and intracellular H^+ affinities

Figure 6. Amiloride-inhibitable $^{22}Na^{+}$ influx of the TM4 single-cysteine substituted mutants in the presence of 10mM MTSES (A) or 1mM MTSET (B). Results are expressed as a percentage of the uptake catalyzed by each mutant in the absence of the MTS compounds. Each bar is representative of an average of at least three experimental trials (± S.E.).

A


MTSES
Amiloride-inhibitable $^{22}Na^+$ Influx (% of Control)
0
20
40
60
80
100
120
140
160
180
200
NHE1"HA
ΔC
L167C
F168C
P171C
D176C
G178C
Y179C

B

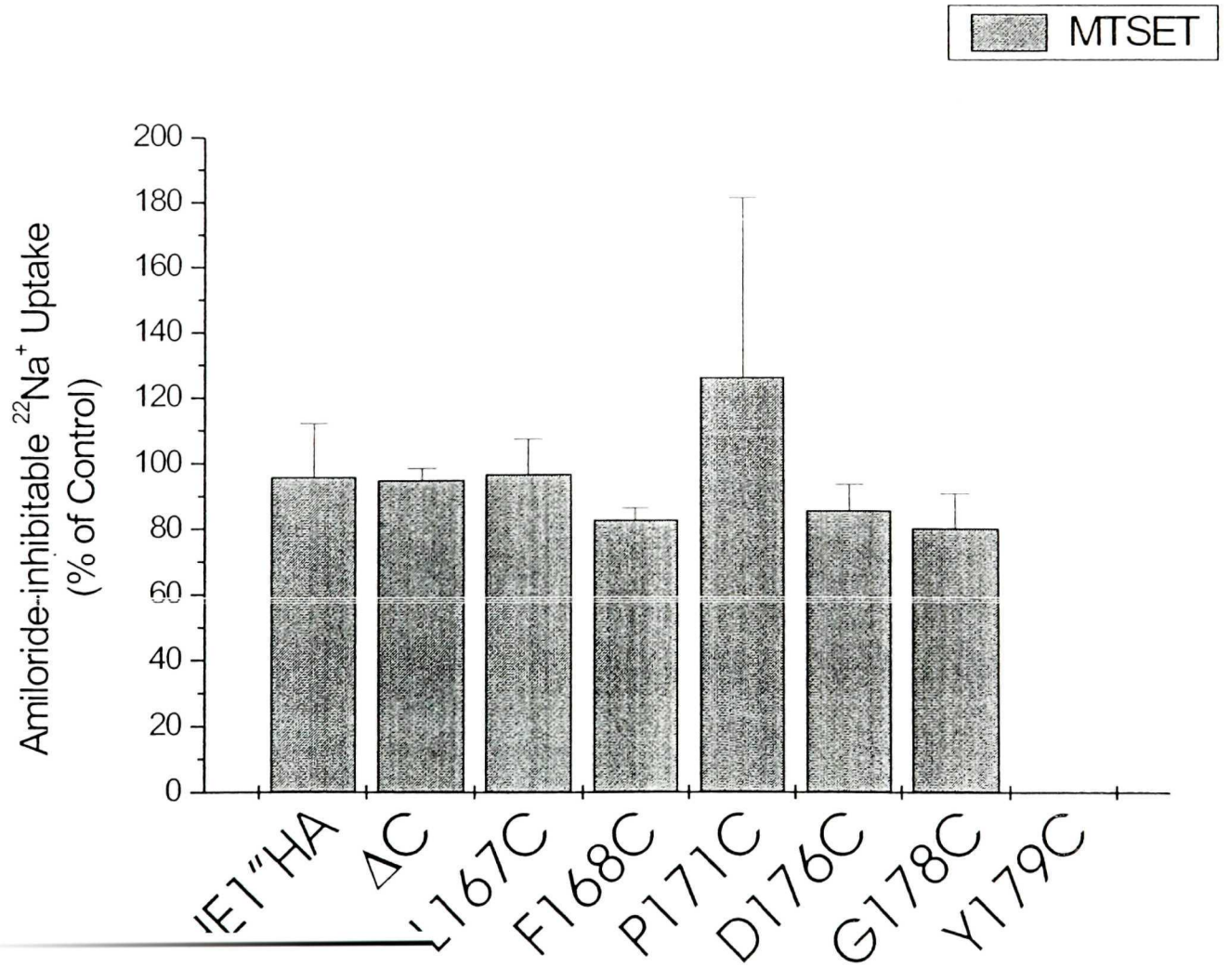
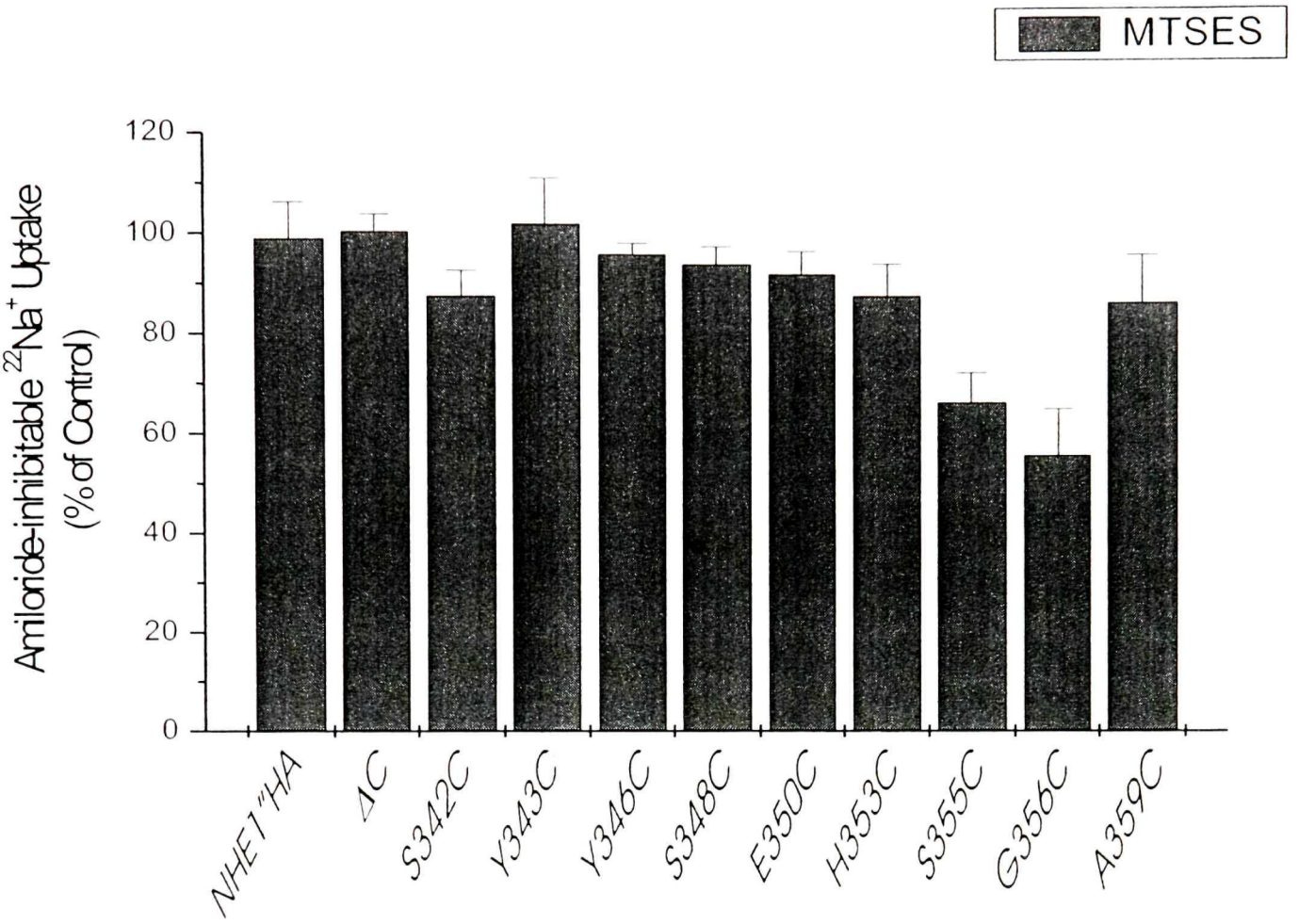
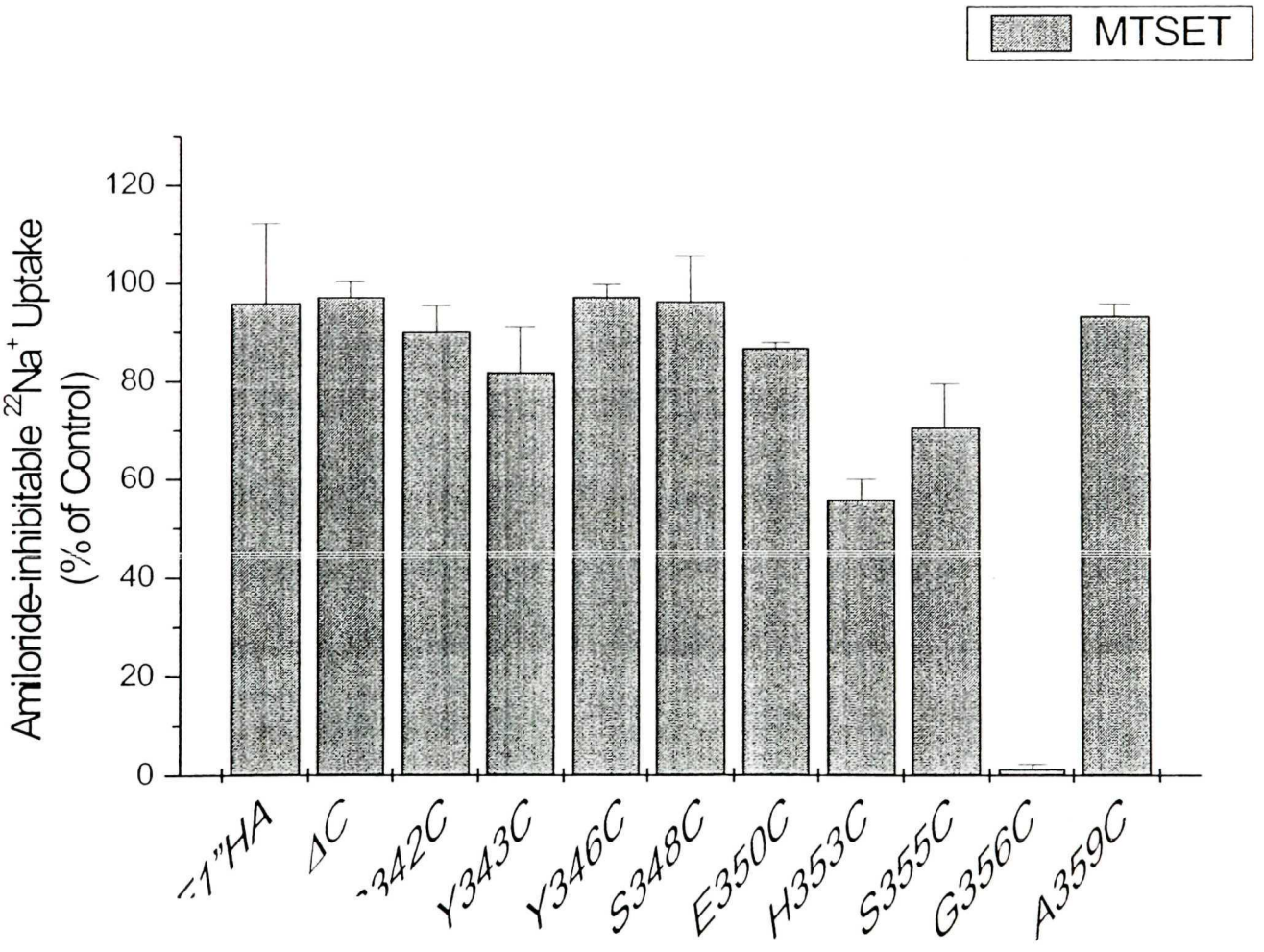

MTSET
Amiloride-inhibitable $^{22}Na^+$ Uptake (% of Control)
0
20
40
60
80
100
120
140
160
180
200
'E1"HA
ΔC
'L167C
F168C
P171C
D176C
G178C
Y179C

Figure 7. Amiloride-inhibitable $^{22}Na^{+}$ influx through the TM9 single-cysteine substituted mutants in the presence of 10mM MTSES (A) or 1mM MTSET (B). Results are expressed as a percentage of the uptake catalyzed by each mutant in the absence of the MTS compounds. Each bar is representative of an average of at least three experimental trials (± S.E.).

A


MTSES
Amiloride-inhibitable $^{22}Na^+$ Uptake
(% of Control)
120
100
80
60
40
20
0
NHE1"HA
ΔC
S342C
Y343C
Y346C
S348C
E350C
H353C
S355C
G356C
A359C

B

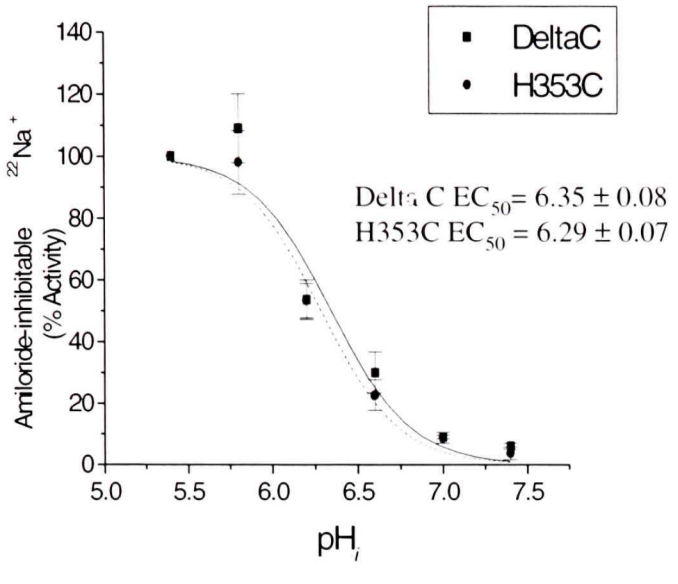

MTSET
Amiloride-inhibitable $^{22}Na^+$ Uptake
(% of Control)
120
100
80
60
40
20
0
Y343C
Y346C
S348C
E350C
H353C
S355C
G356C
A359C

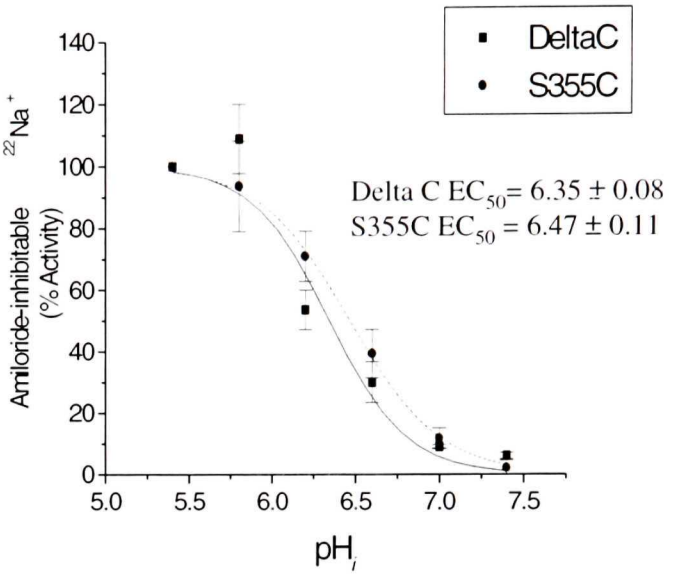
(K_m's) of the mutant exchangers. The P171C mutant was also included in the analysis because of its extremely low rate of transport.

In order to determine the intracellular H^+ affinity, antiport activity was measured by clamping pH_i at specific levels within the range of 5.4-7.4. The pH_i profile of H353C, S355C, and G356C, did not differ significantly from the parental $NHE1_{HA}\Delta C$ construct (Fig. 8). In each case, the exchanger was quiescent when pH_i was clamped at 7.4, with amiloride-inhibitable $^{22}Na^+$ influx increasing progressively up to a maximal level that occurred at pH_i 5.4 (Fig. 8). Results are expressed as a percentage of the amiloride-inhibitable $^{22}Na^+$ influx that occurred at pH 5.4. As displayed by the figure, the apparent H^+ affinity (K_H) of the mutant exchangers did not differ significantly from that determined for $NHE1_{HA}\Delta C$. In contrast, the curve obtained for P171C diverged dramatically from that obtained for the $NHE1_{HA}\Delta C$ (Fig. 9). The activity of the P171C mutant increased more rapidly as a function of the H^+ concentration compared to $NHE1_{HA}\Delta C$, such that it reached maximal activity at ~pH_i 6.25. As a result of this right shift, and the slight transformation of the curve, the K_H for P171C differs significantly from $NHE1_{HA}\Delta C$ ($p < 0.05$) (Fig. 9).

The initial rates of H^+_i-activated $^{22}Na^+$ influx as a function of extracellular Na^+ concentration were examined using solutions containing Na^+ concentrations over a range of 1.25 to 125 mM. The curves obtained were consistent with simple, saturating Michaelis-Menton kinetics as the rates of exchange approached a maximum level with increasing Na^+ concentration in an asymptotic fashion. Calculation of the affinity constants (K_{Na}) for H353C, S355C, and G356C revealed similar values to that determined for $NHE1_{HA}\Delta C$ (Table 1). Although P171C was subjected to this analysis, the K_{Na} was

Figure 8. Intracellular H^+ affinity for H353C, S355C, and G356C. There is no significant difference between the EC_{50}'s calculated for the three mutants compared to that calculated for $NHE1_{HA}\Delta C$, thus suggesting that these residues do not directly bind intracellular H^+. Each value on the graph is a percentage of the activity recorded when intracellular pH was clamped at pH 5.4. Each data point is an average of three experimental trials (± S. E.).


DeltaC
H353C
Delta C EC50= 6.35 ± 0.08
H353C EC50 = 6.29 ± 0.07
22Na+
Amiloride-inhibitable
(% Activity)
pHi


DeltaC
S355C
Delta C EC50= 6.35 ± 0.08
S355C EC50 = 6.47 ± 0.11
22Na+
Amiloride-inhibitable
(% Activity)
pHi

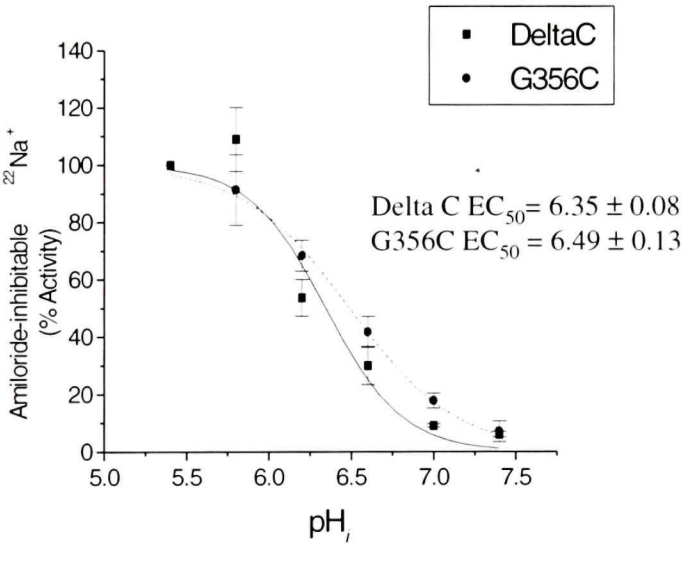

DeltaC
G356C
Delta C EC50= 6.35 ± 0.08
G356C EC50 = 6.49 ± 0.13
22Na+
Amiloride-inhibitable
(% Activity)
pHi

Figure 9. Intracellular H^+ affinity analysis for P171C, a mutation that severely disrupts NHE activity. The curve represents NHE activity as a function of intracellular pH and is characteristically sigmoidal for $NHE1_{HA}$ and $NHE1_{HA}\Delta C$. The curve generated for P171C displays a distinct right shift in its orientation, while its shape is more hyperbolic than sigmoidal. Consistent with these observations, the EC_{50}'s calculated for P171C and $NHE1_{HA}\Delta C$ are significantly different ($p < 0.05$), thus suggesting that this mutation disrupts intracellular H^+ binding to the exchanger. Each data point on the graph is a percentage of the activity recorded when intracellular pH was clamped at pH 5.4. and is representative of three experimental trials (± S. E.).

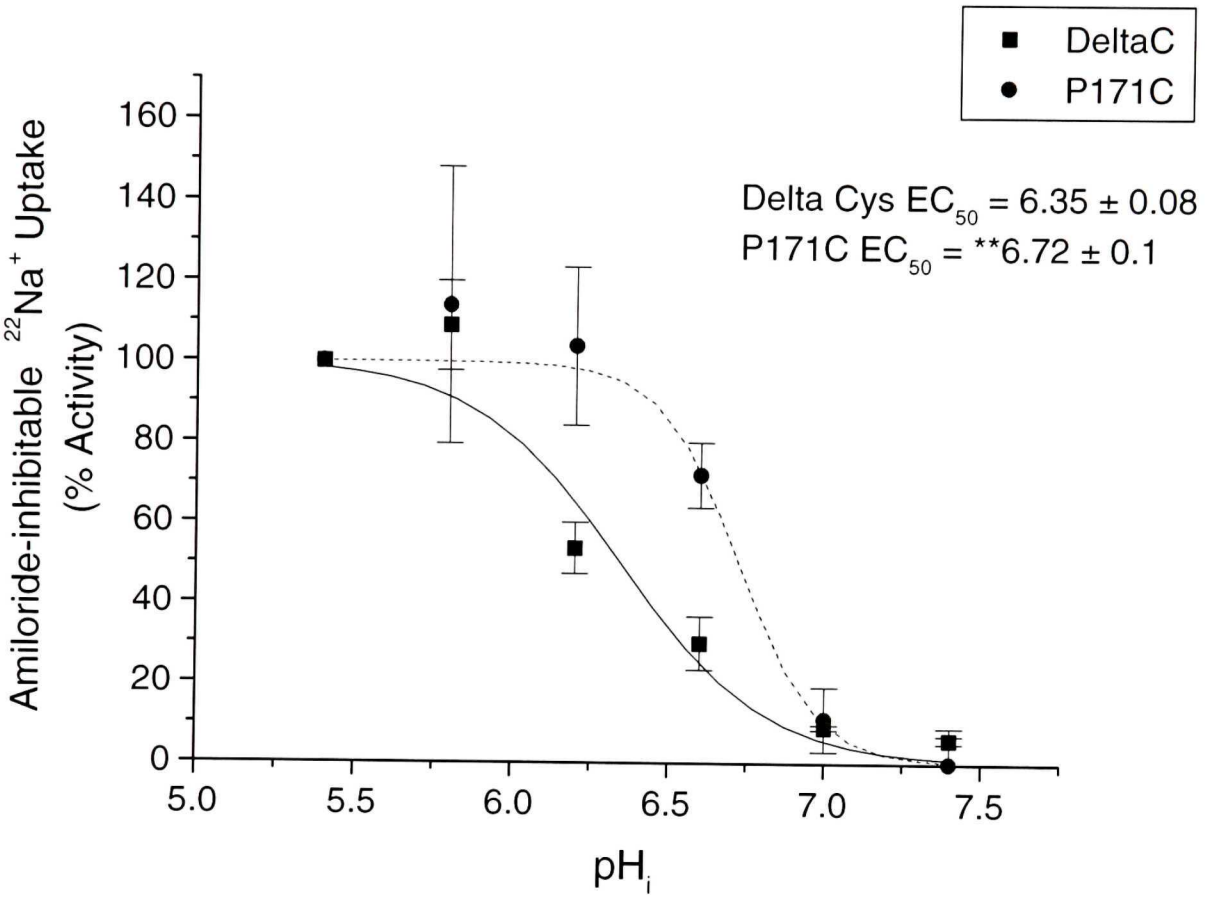

DeltaC
P171C
Delta Cys EC50 = 6.35 ± 0.08
P171C EC50 = **6.72 ± 0.1
Amiloride-inhibitable 22Na+ Uptake
(% Activity)
160
140
120
100
80
60
40
20
0
5.0
5.5
6.0
6.5
7.0
7.5
pHi

Table 1. Experimentally derived K_m's for extracellular Na^+. There was no significant difference in the K_m's determined for the TM9 single-cysteine substituted mutants (H353C, S355C, and G356C) and $NHE1_{HA}\Delta C$. These residues therefore do not appear to bind extracellular Na^+. Calculation of an accurate K_m for P171C was unfeasible due its low counts and inconsistent results between experimental trials. Values are representative of three experiments (± S.E.).

Construct	K_m (mM)	*n*
$NHE1_{HA}\Delta C$	30.1 ± 9.1	3
H353C	41.0 ± 5.6	3
S355C	29.0 ± 7.0	3
G356C	33.3 ± 8.4	3
P171C	N/A	3

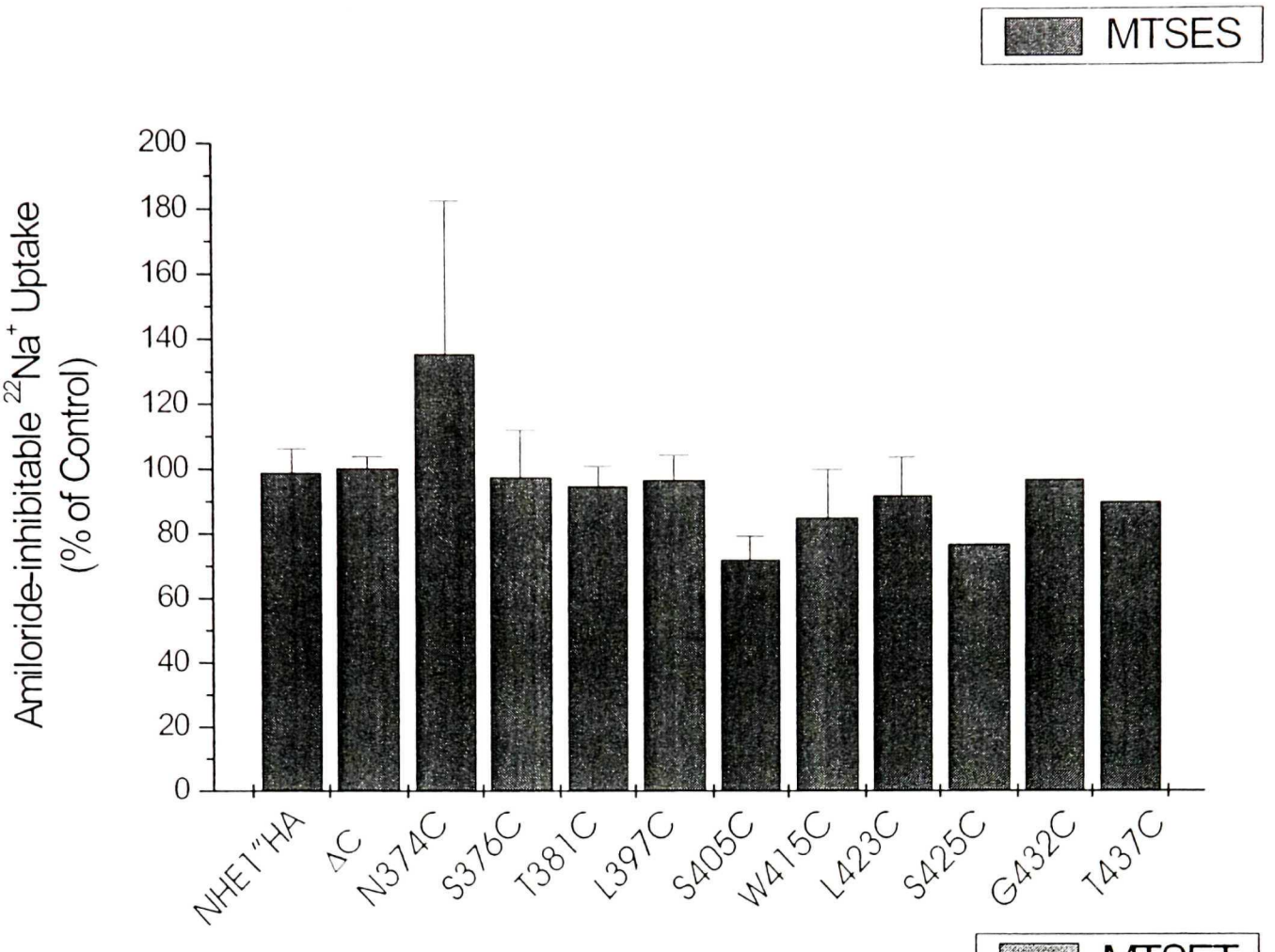
extremely difficult to measure accurately since the activity of this mutant was at the lower limits of detection for this particular radiotracer flux assay. Unlike measurements of transport activity ($^{i.e.}$, $^{22}Na^+$ influx) as a function of intracellular pH, the specific activity of the $^{22}Na^+$ tracer is decreased as the extracellular NaCl concentration increases, which compromises the ability to detect intracellular accumulation $^{22}Na^+$ uptake when the flux rates are low.

MTS Inhibition of the R-Loop and TM10 Single-Cysteine Mutants: To further define sites involved in ion translocation by NHE1, we extended our analyses to the adjacent putative R-Loop and TM10. Structurally, the proposed R-loop resembles the pore-loops (P-loops) described for ion channels, where they have been implicated in ion selectivity and translocation. Hence, it was of interest to ascertain whether this structural element performs a similar function in NHE1.

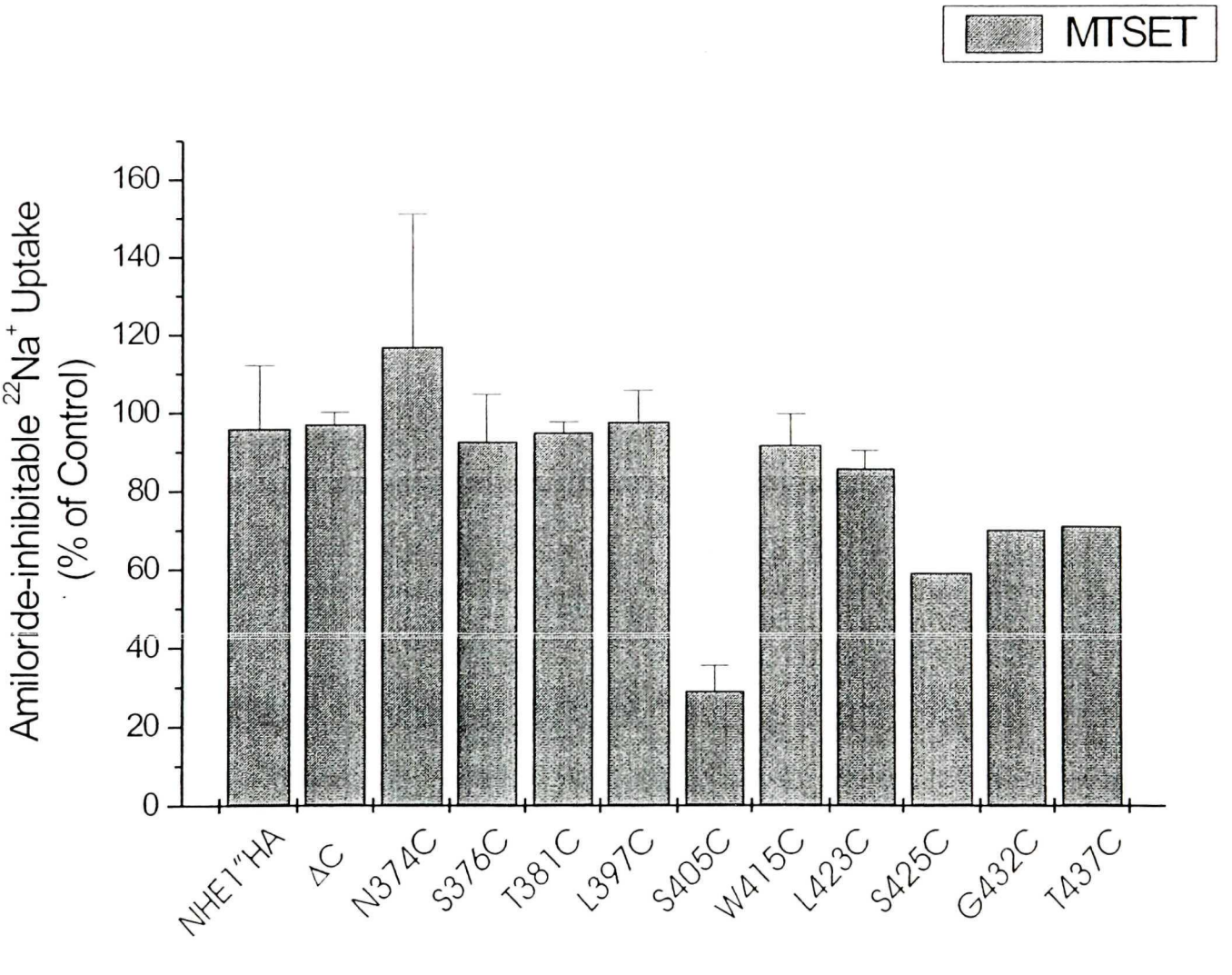
To this end, single-cysteine substituted mutations were made throughout the R-Loop (N374, S376, T831, L397, S405 and W415) and TM10 (L423, S425, G432, T437) and tested for their sensitivity to inhibition by MTS reagents. At shown in Fig. 10, cells expressing the S405C mutant exhibited a dramatic reduction (~ 70%) in $^{22}Na^+$ influx in the presence of MTSET (Fig. 10b). MTSES modification of this same site also caused significant inhibition, although to a lesser extent (Fig. 10a). By contrast, the other R-loop single-cysteine substituted mutants were largely unaffected by the presence of the MTS reagents, although the N374C mutant had a tendency for increased activity in the presence of both MTSES and MTSET. However, the data for this particular mutant were unusually variable and not statistically significant. In TM 10, the S425C, G432C and

ure 10. Amiloride-inhibitable $^{22}Na^{+}$ influx of the single-cysteine mutants of the oop/TM10 in the presence of 10mM MTSES (A) or 1mM MTSET (B). Results are ıressed as a percentage of the uptake catalyzed by each mutant in the absence of the 'S compounds. Each bar is representative of an average of at least three experimental ls (± S.E.). Analysis on S425C, G432C, and T437C has only been performed once, l therefore no standard error could be calculated for these mutants.

A


MTSES
Amiloride-inhibitable $^{22}Na^+$ Uptake (% of Control)
200
180
160
140
120
100
80
60
40
20
0
NHE1"HA
ΔC
N374C
S376C
T381C
L397C
S405C
W415C
L423C
S425C
G432C
T437C

B


MTSET
Amiloride-inhibitable $^{22}Na^+$ Uptake (% of Control)
160
140
120
100
80
60
40
20
0
NHE1"HA
ΔC
N374C
S376C
T381C
L397C
S405C
W415C
L423C
S425C
G432C
T437C

T437C mutants were inhibited ~ 30-40% by MTSET, but this effect was less evident in the presence of MTSES.

With respect to the above analyses, it is important to note that S425C, G432C, and T437C have only been tested once and therefore these data constitute a preliminary result for which no standard error could be calculated. It is also worth mentioning that at least 14 other mutations have been made in the R-loop/TM10 region, but have yet to be analyzed. When completed, the analyses should provide a more comprehensive assessment of the involvement of the R-loop/TM10 region in cation translocation.

DISCUSSION

The objective of this study was to delineate some of the structural elements of NHE1 involved in cation translocation. To this end, the SCAM approach was employed whereby we systematically mutated residues within segments TM4, TM9, and R-loop/TM10. Both TM4 and TM9 were initially targeted as promising regions based on previous studies of the drug-binding properties of the Na^+/H^+ exchangers. Amiloride and its analogues have been characterized as simple competitive (L'Allemain *et* al., 1984; Paris and Pouyssegur, 1983) or mixed-type (Ives *et al.*, 1983, Warnock *et al.*, 1988) inhibitors of extracellular Na^+ transport through the NHEs. Collectively, it was therefore predicted that drugs such as amiloride contact multiple sites on the protein, with at least one or more of these sites coinciding with cation binding. Recent investigations by Khadilkar *et al.* (2001) and others (Counillon *et al.*, 1997; Wang *et al.*, 1995; Counillon *et al.*, 1993a; Orlowski, 1993) partially confirmed this conjecture as most sites identified in both TM4 and TM9 that conferred amiloride sensitivity to NHE1 did not affect Na^+ or

H^+ affinities. While these sites may not be directly involved in the kinetics of cation binding, they may nevertheless still form part of the cation translocation pathway through the membrane. Recently, one site in TM4 (Phe^{166} of rat NHE1) was shown to appreciably reduce Na^+ affinity (Touret *et al.*, 2001). Taken together, both TM4 and TM9 are promising regions for cysteine-scanning mutagenesis in the hope of identifying sites that form part of the cation translocation pathway.

Previous pharmacological analysis of TM4 specifically identified Phe^{165}, Phe^{166}, Leu^{167}, and Gly^{178} as sites critical for amiloride recognition (Khadilkar *et al.*, 2000; Counillon *et al.*, 1997; Counillon *et al.*, 1993a). Leu^{167} and Gly^{178} were therefore among those sites targeted in $NHE1_{HA}\Delta C$ for cysteine substitution mutagenesis. Of the single-cysteine substituted mutants, only Y179C failed to survive selection. Western blotting analysis demonstrated that this protein is not only poorly expressed in the cell, but appears to remain in the immature, core-glycosylated form (Fig. 4a). Interestingly, the tyrosine at this position is 100% conserved across the NHE isoforms, thus suggesting the importance of this residue for protein structure and/or function. Mutation of this residue appears to undermine appropriate protein processing, thus explaining why only the faster migrating band is visible.

In addition, mutation of another residue in this region, P171C, also greatly reduced NHE1 activity. Unlike Y179C, fully glycosylated P171C is detected in AP-1 cells (Fig. 4a). This suggests that the mutation may disrupt the proper functioning of the mature form of the protein in addition to its effects on protein processing. Mutation of the adjacent residue, P172, also dramatically decreases transport activity, again reiterating the importance of this domain (unpublished data). Chemically, the secondary

imino group of a proline residue is held in a rigid conformation that is known to reduce polypeptide flexibility at that point, thereby disrupting the formation of α-helices and β-sheets. Furthermore, Touret *et al.* (2001), hypothesized that the region one helix turn *N*-terminal to this doublet of prolines would be unstable due to a lack of hydrogen-bonding and thus, if not highly flexible, would have to be maintained by a precise set of interactions with other areas of the protein. Additionally, the free backbone carbonyl groups at this site could be involved in the coordination of cations as they translocate through the protein. Collectively, these data suggest the presence of two consecutive proline residues within this segment may form a structurally unique and functionally sensitive region of the protein.

On this background, it was surprising to find that cells expressing the single-cysteine substituted mutants of TM4 did not show any significant reduction in $^{22}Na^{+}$ influx in the presence of the MTS reagents. This suggests that this region of the protein does not directly constitute part of the cation translocation pathway, although one cannot exclude the possibility that these residues were simply not accessible to the MTS reagents from the extracellular side of the membrane. However, the region directly adjacent to it, a putative R-loop between TM4 and TM5, has been implicated in ion exchange in a previous study (Wakabayashi *et al.*, 2000). $^{22}Na^{+}$ influx by two mutations in this domain, R180C and Q181C, was inhibited by externally applied MTSET. However, these residues were not amenable to modification by externally applied biotin maleimide – another thiol-modifying reagent - but could be biotinylated when applied internally. Presumably the large size of this compound precludes its ability to access these resides when applied externally. Taken together, these data suggest that at least part of this loop

is localized within the membrane and has access to both the internal and external milieu of the cell. As such, it was proposed that this loop may form a structure that lines the aqueous pore and is involved in ion transport (Wakabayashi *et al.*, 2000).

The importance of the R-loop between TM4 and TM5 is further emphasized by the knowledge that the geometrically-constrained region comprising TM4 and the adjacent TM4/TM5 R-loop has been previously demonstrated to play a crucial role in sodium binding (Touret *et al.*, 2001). Applying this knowledge to our data suggests a situation in which TM4 facilitates TM4/TM5 R-loop mediated-ion exchange. Specifically, the proline-rich segment of TM4 has been speculated to introduce a kink into the α-helix that would generate sufficient room in the lipid bilayer to accommodate the R-loop between TM4 and TM5 (Wakabayashi *et al.*, 2000). As such, perturbations in the structural configuration of TM4, like the mutation of highly conserved residues like Pro^{171} or Tyr^{179}, may disrupt the spatial positioning of the TM4/TM5 R-loop, thereby compromising exchanger activity, as demonstrated in this study. Furthermore, since TM4 contains residues that contribute to amiloride sensitivity, a distinct possibility could be that multiple residues in this area co-operate to yield a non-competitive drug-binding pocket.

Another functionally significant role for TM4 may be pH_i sensing. It has been proposed that allosteric H^+ regulatory site(s) exist in NHE1 which synergistically interact with the translocation site to promote rapid extrusion of H^+ once intracellular pH falls below a certain threshold level (Szabo *et al.*, 2000; Aronson, 1982). According to the kinetic data obtained for P171C, TM4 may either influence or directly encompass one of these sites. Despite a dramatic decrease in its intrinsic catalytic turnover rate, P171C

exhibited a significant increase in its sensitivity to intracellular pH. Thus, TM4 may constitute part of the H^+-modifier site, a function that is compromised by the P171C mutation.

In addition to TM4, TM9 also contains residues of pharmacological significance. Specifically, Glu^{350} and Gly^{356} are two residues that have been demonstrated to influence amiloride sensitivity of NHE1. Therefore, these two residues were among those targeted for single-cysteine substituted mutagenesis. Unlike TM4, specific single-cysteine substituted mutants within TM9 were inhibited in the presence of the MTS reagents. In particular, the activity of G356C was almost completely inhibited (~ 98.5%) in the presence of positively-charged MTSET (Fig. 7b). Likewise, transport activity was also significantly reduced by negatively-charged MTSES, although to a considerably lesser extent (~ 40%). These data indicate that the amino acid side chain at this position – in this case a thiol group - faces the aqueous milieu and is readily accessible for covalent modification by the MTS reagents. Mechanistically, thiol-modification of cysteine-356 may block cation transport by sterically hindering critical conformation changes that occur during cation translocation, either at the cation pore itself or at a more distant site that nevertheless impacts on cation translocation. However, the observed sizeable differences in the degree of inhibition elicited by the two oppositely-charged, but similarly-sized, MTS reagents also implicates the involvement of electrostatic forces on the flow of cations through the protein, suggesting that residue 356 is more likely in close proximity to the cation translocation pathway. Thus, aside from possible steric effects, the positive charge of MTSET could further impede the flow of Na^+ by electrostatic

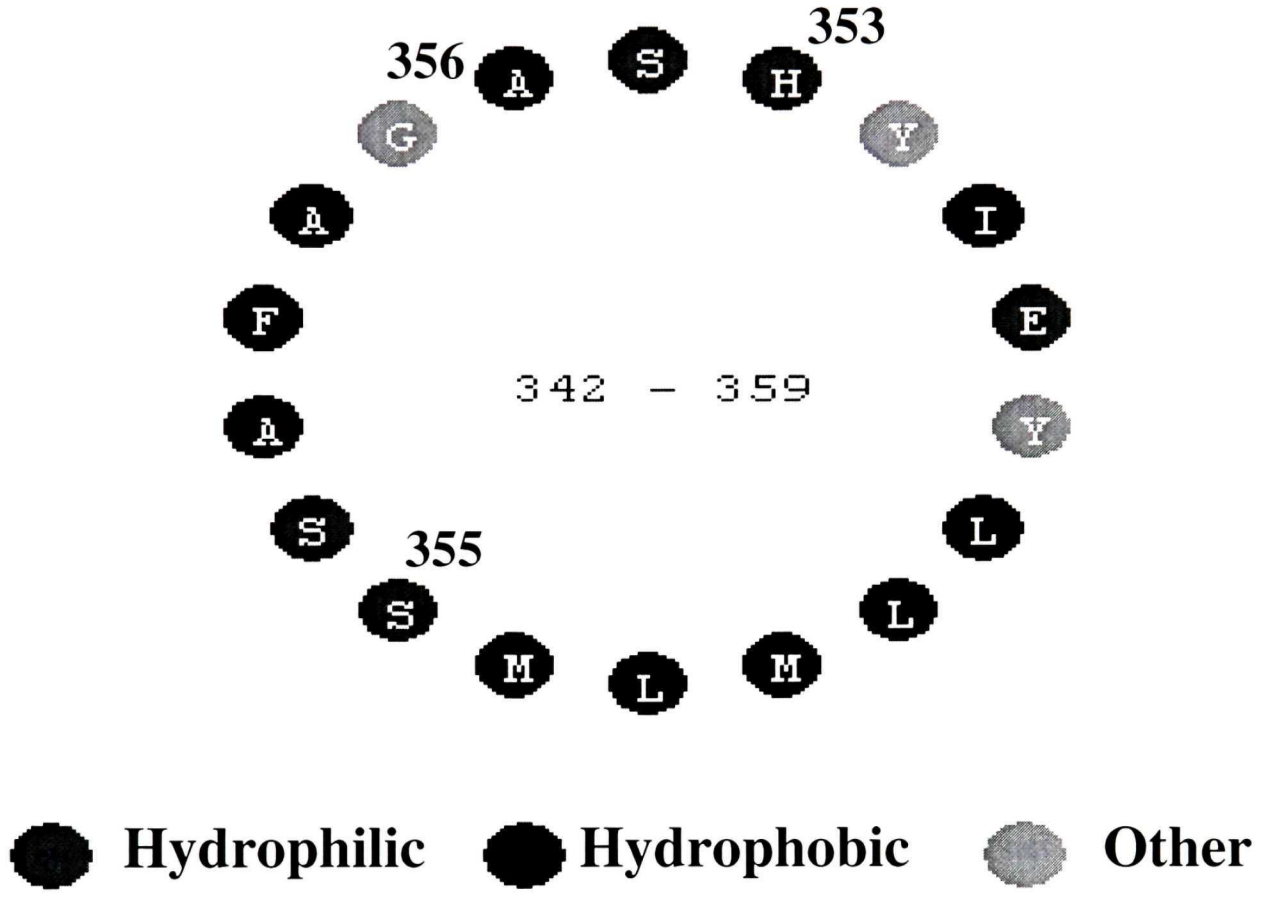
repulsion, whereas the negatively-charged MTSES reagent could act as an attractant and retard passage of Na^+ ions through the pore, but with less efficiency than MTSET.

In addition to G356C, cysteine-substitution of two adjacent N-terminal residues, H353C and S355C, also renders the transporter moderately sensitive to inhibition by the MTS reagents, further supporting a role for this segment as part of the pore-lining. The reduction in the degree of MTS-mediated inhibition might be expected as these residues are predicted to lie deeper within the membrane and their thiol side-chains would be directed more towards the lipid bilayer, assuming 3.6 amino acids per turn of an α-helix relative to position 356, making them less available for modification by the MTS reagents (Fig. 11). While H353 and S355 are hydrophilic residues, it is perhaps initially surprising to find that a hydrophobic residue such as G356 would also directly face the aqueous pore. However, since glycine contains a hydrogen atom as its side-chain group, it is only moderately nonpolar. Moreover, previous studies have demonstrated that hydrophobic residues line the translocation pores of the anion exchanger isoform 1 (AE1) (Tang *et al.*, 1998), the γ-aminobutyric acid type A receptor (Xu and Akabas, 1993), and the potassium channel (Doyle *et al.*, 1998). It has been theorized that hydrophobic residues are the most energetically favorable amino acids to have lining a pore as they would provide an inert surface for the diffusing ion. Otherwise, the wall of the pore would impede the ion as it traverses the pore, thus lowering the turnover rate and efficiency of exchange (Doyle *et al.*, 1998). Taken together, these data are consistent with the notion that TM9 is part of the cation translocation pore.

Having implicated these three residues in forming part of the pore-lining, we were next interested in assessing whether they are also directly involved in cation recognition

Figure 11. Helical wheel projection of TM9. Note that His^{353} and Ser^{355} flank Gly^{356} (which is almost completely inhibited by MTSET) in an α–helix. All three amino acids are located in relative proximity to each other on the more hydrophilic/neutral face of the helix and are thus likely candidates to face the aqueous pore.

5YMAYLSAELFHLSGIMA



and binding. Kinetic analyses, however, did not indicate any shift in the affinities for extracellular Na^+ or intracellular H^+ of H353C, S355C, or G356C (Fig. 8a, Table 1), suggesting that these residues *per se* are not critical determinants of Na^+ or H^+ binding. However, it is clear that these residues constitute an important structural element of the cation translocation pathway, and, as such, may participate in other components of ion exchange. One possibility is that the Gly^{356} may be a focal point for specific conformational changes that facilitate cation translocation. Glycine, the simplest amino acid, provides minimum steric hindrance to rotation of the polypeptide chain. MTS-catalyzed addition of a large, covalent side chain to G356C may deprive the protein of the necessary flexibility at this point, and cation exchange would be impeded.

An equally plausible interpretation of the results could be that H353C, S355C, and G356C are simply elements that line the cation translocation pathway or pore. In this case, the data suggests that the pore is aligned as an outwardly facing funnel that narrows to a permeability barrier following H353. This might explain why MTS reagents have no effect on NHE1 mutants containing cysteine-substitutions below H353 (*i.e.*, S342C-E350C). The MTS compounds may only fit into cylinders that are 6 Å in diameter and 10 Å in length (Karlin and Akabas, 1998). Hence, if the pore does not adhere to these dimensions after His^{353}, the MTS compounds will not be able to penetrate beyond this point, rendering residues below this point inaccessible. Such pore configurations are not uncommon, as they have been described for the FMRF-amide activated sodium channel (a member of the epithelial Na^+ channel/Degenerin gene family) (Poet *et al.*, 2001), AE1 (Tang *et al.*, 1998), and the KcsA K^+ channel (Doyle *et al.*, 1998).

Unification of the pharmacological and MTS data suggests that TM9 constitutes at least part of the competitive drug-binding pocket. Gly^{356}, a residue previously implicated in antagonist binding, has now been demonstrated to also influence cation translocation. Therefore, an attractive explanation is that this constitutes part of the competitive drug-binding pocket as both cations and antagonists would be competing for access to this region.

Having established TM9 as an important structural element in the cation translocation pathway, the adjacent putative R-loop and TM10 were investigated next. The R-loop shares structural similarities to P-loops that have previously been demonstrated to fulfill a role in ion flow through channels and pumps (Li *et* al., 2000; Balser, 1999; Schneider and Scheiner-Bobis, 1997). It is important to note that ion channels and pumps do differ from ion exchangers in a number of respects. For instance, ion channels are thought to consist of a hydrophilic pore through which specific inorganic ions are allowed to permeate through, exclusive of the need to bind specific carrier protein residues. As well, other than the opening and closing of the gates, no conformational changes facilitating transport are thought to occur in ion channels. Ion pumps, while similar to ion exchangers, require ATP to initiate transport activity. However, despite these differences, the potential exists that certain structural elements may be conserved between these three types of membrane transporters. The fact that ion channels and pumps, similar but distinct membrane transport proteins, both share P-loops certainly endorses this inference (Li *et* al., 2000; Balser, 1999; Schneider and Scheiner-Bobis, 1997).

By definition, P-loops are hydrophilic extracellular stretches of ion channels that can invaginate into the membrane bilayer where they are surrounded by the hydrophobic transmembrane segments of the protein (Schneider and Scheiner-Bobis, 1997). These structural elements are responsible for mediating antagonist binding, ion selectivity, and conductance. They are also involved in dictating the ion specificity of the protein, as permeation properties conferred by certain P-loop sequences can be transferred between channels using chimeric mutational analysis (Balser, 1999).

Preliminary MTS analysis of the single-cysteine substituted mutants of the R-loop/TM10 has identified Ser^{405} as an important contributor to ion translocation. MTSET modification of S405C inhibits $^{22}Na^{+}$ influx through the protein by approximately 70% (Fig. 10b). Therefore, it appears as if the R-loop between TM9 and TM10 may function in similar fashion in ion exchangers. However, additional mutagenesis in this region is required to confirm these results. To this end, additional residues have been identified for single-cysteine substituted mutagenesis (Fig. 2). In particular, Glu^{395} has been targeted because it is highly conserved amongst the isoforms, negatively-charged, and in close proximity to Ser^{405}.

In summary, residues in TM9 and the R-loop/TM10 comprise important structural elements for cation translocation in NHE1. Specifically, His^{353}, Ser^{355}, Gly^{356} and Ser^{405}, have all been identified as residues that contribute to the exchange mechanism of NHE1. Of these residues, Ser^{355}, Gly^{356} and Ser^{405} are highly conserved amongst the members of the NHE gene family, further supportive of their functional importance. More analysis will be required for the putative R-loop between TM4 and TM5 and the region encompassing R-loop/TM10. In addition, the intracellular loop between TM8 and TM9

displays similar topological characteristics as the TM4/TM5 loop and therefore may be another good candidate region for study (Wakabayashi *et al.*, 2000). Neutral MTS reagents, MTSEH (-OH group) and MTSBN (-phenyl group) can be applied to distinguish between the electrostatic and steric components of MTS-inhibition, while weakly membrane-permeant MTS reagents such as MTSEA could be employed to further deduce pore structure. Finally, should MTS screening of single-cysteine substituted mutants in the TM4/TM5 R-loop prove encouraging, attempts should be made to cross-link this area to TM9 and the R-loop/TM10 in order to demonstrate their close proximity to each other as constituents of the putative pore.

A growing need to reveal the underlying mechanisms that govern NHE function has been gaining momentum from both an academic and therapeutic standpoint. Integration of the results of the current and proposed studies should have profound implications for this endeavor. Aside from elucidating the underlying mechanisms of cation exchange in the NHEs, these studies should further define the means by which the *C*-terminal cytoplasmic tail regulates ion translocation through the *N*-terminal transmembrane domain. Finally, it is anticipated that development of more efficacious, NHE-specific therapeutic agents will be promoted by delineation of the various structural domains of the exchanger.

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