Topology of NBCe1 Protein Transmembrane Segment 1 and Structural Effect of Proximal Renal Tubular Acidosis (pRTA) S427L Mutation*

Received for publication, July 24, 2012, and in revised form, January 4, 2013. Published, JBC Papers in Press, January 28, 2013, DOI 10.1074/jbc.M112.404533

Quansheng Zhu‡†, Weixin Liu‡, Liyo Kao‡, Rustam Azimov§, Debra Newman‡, Natalia Abuladze‡, and Ira Kurtz†‡§

From the ‡Department of Medicine and §Brain Research Institute, David Geffen School of Medicine, UCLA, Los Angeles, California 90095-1689

Background: NBCe1-A-TM1 is involved in forming part of the ion permeation pathway.

Results: NBCe1-A-TM1 contains 31 amino acids. The pRTA S427L mutation alters the normal aqueous accessibility of specific TM1 residues.

Conclusion: NBCe1-A-TM1 is tilted in the lipid bilayer, and its N terminus interacts with the cytoplasmic domain. The presence of S427L altered NBCe1-A-TM1 orientation.

Significance: Our findings provide novel insights into the pathogenic mechanism of pRTA.

In the kidney proximal tubule, NBCe1-A plays a critical role in absorbing HCO₃⁻ from cell to blood. NBCe1-A transmembrane segment 1 (TM1) is involved in forming part of the ion permeation pathway, and a missense mutation S427L in TM1 impairs ion transport, causing proximal renal tubular acidosis.

In the present study, we examined the topology of NBCe1-A-TM1 in detail and its structural perturbation induced by S427L. We analyzed the N-terminal cytoplasmic region (Cys-389–Gln-424) of NBCe1-A-TM1 using the substituted cysteine scanning accessibility method combined with extensive chemical stripping, in situ chemical probing, and functional transport assays. NBCe1-A-TM1 was previously modeled on the anion exchanger 1 TM1 (AE1-TM1); however, our data demonstrated that the topology of AE1-TM1 differs significantly from NBCe1-A-TM1. Our findings revealed that NBCe1-A-TM1 is unusually long, consisting of 31 membrane-embedded amino acids (Phe-412 to Thr-442). The linker region (Arg-394–Pro-411) between the N terminus of TM1 and the cytoplasmic domain is minimally exposed to aqueous and is potentially folded in a helical structure that intimately interacts with the NBCe1-A cytoplasmic domain. In contrast, AE1-TM1 contains 25 amino acids connected to an aqueous-exposed cytoplasmic region. Based on our new NBCe1-A-TM1 model, Ser-427 resides in the middle of TM1. Leucine substitution at Ser-427 blocks the normal aqueous exposure to Thr-442, Ala-435, and Lys-404, implying a significant alteration of NBCe1-TM1 orientation. Our study provides novel structural insights into the pathogenic mechanism of S427L in mediating proximal renal tubular acidosis.

The Na⁺–HCO₃⁻ cotransporter, NBCe1-A, is responsible for absorbing the filtered HCO₃⁻ in the kidney proximal tubule from cell to blood (1, 2). It electrogenically transports Na⁺ and HCO₃⁻ against their respective concentration gradients across the basolateral membrane of proximal tubule cells (1). NBCe1-A exists as a homodimer in the lipid bilayer, of which each monomer is functionally independent (3). The N-terminal region of NBCe1-A forms a cytoplasmic domain that was predicted to interact tightly with the C-terminal membrane-embedded transmembrane region (4).

Nonsense and missense mutations in NBCe1-A have been reported that impair the transporter function, causing autosomal recessive proximal renal tubular acidosis (pRTA)² with ocular and intracerebral abnormalities (5–9). We recently determined that NBCe1-A contains 14 transmembrane segments (TM) and that the residues mutated in pRTA (R298S, S427L, T485S, G486R, R510H, L522P, A799V, and R881C) are located in TM1, -3, -4, -10, and 12, respectively (except R298S located in the cytoplasmic domain) (see Fig. 1A) (10). The pRTA-causing mutation, S427L (located in TM1), does not affect NBCe1-A membrane processing, albeit it was reported to alter the mutant transporter polarity of membrane targeting in Madin-Darby canine kidney cells (11). Functional analysis in the Xenopus oocytes indicated that S427L impairs NBCe1-A transport function severely (6). The disease-causing mechanism of S427L was hypothesized to potentially involve disruption of NBCe1-A voltage sensing (6), affecting Na⁺ coordination (6) or the local conformation of the cotransporter (12).

Interestingly, when Ser-427 was substituted with Thr, Ala, or Cys, NBCe1-A retained substantial transport activity (>50%) (6, 10, 12).

The abbreviations and trivial names used are: pRTA, proximal renal tubular acidosis; TM, transmembrane segment; AE1, anion exchanger 1; SCSAM, substituted cysteine scanning accessibility method; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl) methanethiosulfonate; streptavidin-biotinylated-horseradish peroxidase complex; SAO, Southeast Asian ovalocytosis; ER, endoplasmic reticulum.

2 The abbreviations and trivial names used are: pRTA, proximal renal tubular acidosis; TM, transmembrane segment; AE1, anion exchanger 1; SCSAM, substituted cysteine scanning accessibility method; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl) methanethiosulfonate; streptavidin-biotinylated-horseradish peroxidase complex; SAO, Southeast Asian ovalocytosis; ER, endoplasmic reticulum.
The structural and functional importance of the proposed NBCe1-A-TM1 has been previously analyzed by the substituted cysteine scanning accessibility method, and 3 residues (Thr-442, Ala-435, and Ala-428) were detected to be involved in forming the ion permeation pathway (13). Specifically, cysteine-substituted Thr-442 and Ala-435 are sensitive to the inhibition of extracellular methanethiosulfonate (MTS) reagents, and Ala-428 is sensitive to the inhibition of intracellular MTS reagent. Thr-442 is located at the C-terminal extracellular end of TM1, and the function of its cysteine substitution is fully blocked by the smallest MTS reagent, MTSEA, indicating that it may be involved in forming a narrow gate for the entrance of the transported ions (13). The topology of NBCe1-A-TM1 was previously proposed based on the human anion exchanger 1 (AE1) (see Fig. 1B), and indeed, the C-terminal extracellular end of NBCe1-A-TM1 strongly matches that of AE1-TM1 (13, 14). The AE1-TM1 is predicted to consist of 20 amino acids ranging from Gln-404 to Phe-423 (see Fig. 1C). If the N-terminal end of NBCe1-A-TM1 (predicted to start at Gln-424) fully resembles AE1-TM1 topologically, it would be shorter than a standard TM that is composed of 19 amino acids. The extreme C-terminal transmembrane region of NBCe1-A was recently determined to fold differently from AE1 and more closely resembles AE1-TM1 by the substituted cysteine scanning accessibility method. Our findings indicate that NBCe1-A-TM1 is significantly longer than previously thought and that S427L significantly alters the orientation of TM1. Given the importance of TM1 in NBCe1-A function, we performed a series of experiments that addressed the following questions. 1) Where is the intracellular border of NBCe1-A-TM1? 2) How is the linker region between TM1 and the cytoplasmic domain folded in the native structure? 3) What is the effect of S427L on the conformation of NBCe1-A-TM1? To precisely compare the topological differences in TM1 between NBCe1-A and AE1, we completely mapped the topology of AE1-TM1 by the substituted cysteine scanning accessibility method. Our findings indicate that NBCe1-A-TM1 is significantly longer than previously thought and that S427L significantly alters the orientation of TM1. Our results provide novel insights into the structural and mechanistic effect of the NBCe1-A-S427L mutation in causing pRTA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Site-directed mutagenesis kits were from Stratagene. Biotin maleimide, BCECF-AM, DMEM, and all cell culture reagents were from Invitrogen. MTSEA and MTS-TAMRA were from Toronto Research Chemicals Inc. [14C]NEM was from PerkinElmer Life Sciences. Protein A-Sepharose, streptavidin/biotinylated-heroserdiaxid peroxidase complex (streptavidin-HRP), and goat anti-rabbit IgG-conjugated horseradish peroxidase were from GE Healthcare. Mouse monoclonal anti-human Band-3/AE-1 antibody (AE12-M) was from Alpha Diagnostics. IGEPA, polyllysine, and nigericin were from Sigma. PVDF membrane was from Millipore.

**Site-directed Mutagenesis**—A modified human NBCe1-A construct (NBCe1-A-5C-546) with 5 endogenous cysteines substituted with serines and a cysteineless human AE1 construct (AE1-C-546) were used as the templates for site-directed mutagenesis (13). 64 amino acids that cover the whole NBCe1-A-TM1 and 55 amino acids that cover the whole AE1-TM1 were individually substituted with cysteines in NBCe1-A-5C-546 and AE1-C-546, respectively. 14 cysteine substitutions in NBCe1-A were constructed previously (13). Mutagenesis was performed using the Stratagene site-directed mutagenesis kit following the manufacturer’s instructions. The complete cDNA sequence of each mutant was verified by DNA sequencing.

**Protein Expression**—Mutant NBCe1-A and AE1 proteins were transiently expressed in the human embryonic kidney 293 cells (HEK 293) by using Lipofectamine 2000 reagent following the manufacturer’s instructions.

**Immunocytochemistry**—Transfected cells (24 h expression) were rinsed with PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) and incubated with a rabbit anti-human NBCe1-A antibody recognizing extracellular loop 3 (Ab-162, 1:100 dilutions in PBS) (13) for 15 min at room temperature. Ab-162 antibody was then removed, and cells were washed twice with PBS and incubated with goat anti-rabbit IgG conjugated with Cy3 (1:500 dilution in PBS, from Jackson ImmunoResearch) for 15 min at room temperature. The stained cells were mounted in Crystal/Mount (from Biomeda, Foster City, CA), and fluorescence images were acquired by a PXL charge-coupled device camera (model CH1; Photometrics) coupled to a Nikon Microphot-FXA epifluorescence microscope.

**Biotin Maleimide Labeling and Immunoprecipitation**—Whole cell labeling with biotin maleimide was performed as described previously (13). Briefly, transfected HEK 293 cells were collected in PBSCM (PBS containing 0.1 mM CaCl2, and 1 mM MgCl2, pH 7.0) and labeled with 0.2 mM biotin maleimide for 10 min at room temperature. Cells were then lysed in 500 μl of IPB buffer (5 mM EDTA, 150 mM NaCl, 1% (v/v) polyethylene glycol, 0.5% (v/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.5) containing 0.2% (v/v) bovine serum albumin and protease inhibitors (from Roche Applied Science), and NBCe1-A protein was immunoprecipitated by a rabbit anti-human NBCe1-A N terminus monoclonal antibody (21).

**SDS-PAGE and Immunoblotting**—Protein samples were resolved on 7.5% SDS-PAGE and transferred to PVDF membranes. Biotinylated proteins were detected by incubation of blots with 1:10000 diluted streptavidin-biotinylated horseradish peroxidase (GE Healthcare) in TBSTB buffer (TBST buffer (0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5), con-
Transmembrane Segment 1 of NBCe1-A

taining 0.5% (w/v) bovine serum albumin). Protein expression level was assessed by probing of the stripped blots with an anti-NBCe1 C terminus polyclonal antibody (13).

Transport Assay of NBCe1-A—Transport assays were performed as described previously (13). Briefly, transfected cells were loaded with the pH probe BCECF-AM and were then equilibrated in HEPES-buffered Na+–free solution (140 mM tetramethyl ammonium chloride, 2.5 mM K2HPO4, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 5 mM HEPES, pH 7.4) for 25 min. Subsequently, intracellular pH was acutely decreased by exposing cells to a HCO3–-buffered Na+–free solution (115 mM tetramethyl ammonium chloride, 2.5 mM K2HPO4, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 25 mM TMAHCO3, pH 7.4). When the base line was stabilized, cells were exposed to a HCO3–-buffered Na+–containing solution (115 mM NaCl, 2.5 mM K2HPO4, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 25 mM NaHCO3, pH 7.4). EIPA (15 μM) was included in all assay solutions to block endogenous Na+–H+ exchange activity. The rate of pHi recovery, dpH/dt, was calculated in the initial 15 s following exposure to Na+. For transport assays with MTSCE, cells were exposed to MTSEA (1 mM for 1 min) in a HEPES-buffered Na+–free solution (without EIPA) prior to the Na+-induced flux measurements. Transport function of mutant proteins was depicted as a percentage of NBCe1-A-5C− flux for comparison, and sensitivity of mutants to MTSEA treatment was depicted as a percentage of activity in the absence of MTSEA. Each assay was performed at least three times.

Whole Cell MTS-TAMRA Labeling—Transfected HEK 293 cells were washed three times with PBS and incubated with 100 μM MTS-TAMRA (1:1000 dilution in PBS, pH 7.4) for 3 min on ice. The cells were then washed twice with PBS and imaged using an excitation wavelength of 545 nm and an emission wavelength of 590 nm (Olympus BH2 microscope).

MTS-TAMRA Labeling of Isolated Plasma Membranes— Transfected HEK 293 cells were homogenized with a Dounce homogenizer in homogenization buffer (10 mM Tris, 5 mM EDTA, pH 7.4) containing protease inhibitors (from Roche Applied Science). Cell membranes were obtained after a low speed (4,000 × g, 5 min, 4 °C) and a high speed centrifugation (35,000 × g, 30 min, 4 °C). Membrane pellets were then resuspended in PBS (pH 7.5) and labeled with 0.1 mM MTS-TAMRA (50 mM stock in dimethyl sulfoxide (DMSO)) for 20 min at room temperature. The reaction was terminated by adding 5-fold molar excess of DTT. The membranes were then pelleted, and the samples were processed for immunoprecipitation as described previously (4). Protein samples were eluted with a 2× sample buffer without β-mercaptoethanol, resolved on SDS-PAGE, and transferred to PVDF membranes. The dried PVDF membranes were analyzed with a Typhoon 9410 scanner using an excitation wavelength of 545 nm and an emission wavelength of 590 nm.

[14C]NEM Labeling of Isolated Plasma Membranes—Assays were performed following Ref. 22. Briefly, isolated cell membranes were resuspended in 50 μl of PBSCM (pH 7.0) and labeled with 0.5 mM [14C]NEM for 30 min at room temperature. The reaction was quenched by adding 5-fold molar excess DTT, and membranes were collected by a high speed centrifugation for 30 min (35,000 × g, 4 °C). The membrane pellet was then lysed in 500 μl of IPB buffer and immunoprecipitated with an anti-NBCe1-A N-terminal antibody as described previously (4). Protein samples were resolved on 7.5% SDS-PAGE and transferred to PVDF membranes. The dried membranes were exposed to Kodak Bio-X film for 14 days at −80 °C and then probed with an anti-NBCe1-A C-terminal antibody.

Plasma Membrane Stripping—Isolated cell membranes were resuspended in 100 μl of 0.3 M sucrose, mixed with 2 ml of ice-cold 100 mM Na2CO3 (pH 11.5), and incubated for 30 min on a rotating shaker at 4 °C. Membranes were then collected by high speed centrifugation, washed twice with PBSCM, and resuspended in 1.0 ml of PBSCM followed by biotin maleimide labeling.

Image and Data Analysis—Films from immunoblots, biotinylation blots, and [14C]NEM blots were scanned with a Hewlett-Packard Scanjet 5590. Scanned images were quantified with UN-SCAN-IT gelTM Version 6.1 software. Biotinylation and [14C]NEM incorporation levels were calculated according to Ref. 23.

Statistical Analysis—Means ± S.E. were calculated with SigmaPlot 10 software. Statistical analysis was performed using SigmaPlot 10 software. Dunnet’s t test was used to assess statistical significance with p < 0.05 considered significant.

RESULTS

Topological Determination of NBCe1-A-TM1

Construction and Cellular Location of NBCe1-A-substituted Cysteines—To determine the topology of NBCe1-A-TM1 in the cellular environment, we individually substituted 64 amino acids between Cys-389 and Asp-452 with cysteines in NBCe1-A-5C− that covers the entire predicted NBCe1-A-TM1 and the linker region between TM1 and the cytoplasmic domain (Fig. 1B). Cysteine substitutions between Asn-439-Asp–452 were constructed previously (13). NBCe1-A-5C− is a modified NBCe1-A with 5 endogenous cytoplasmic cysteines substituted with serines (Cys-120, -389, -399, -992, -1035) and is free of endogenous reactive cysteines and fully functional (13).

Substitution of a single amino acid in NBCe1-A may misfold the protein, causing endoplasmic reticulum (ER) retention (4). To test whether cysteine substitution in TM1 affects NBCe1-A membrane processing or protein synthesis, we performed immunocytochemistry and Western blot analysis on each of the cysteine-substituted TM1 constructs in the HEK 293 cells. 24 h after transfection, cells were washed and incubated with an antibody that specifically recognizes the NBCe1-A extracellular loop 3 (Ab-162). The results showed that cysteine substitution of Asp-405, Lys-409, Asp-416, Gln-424, Tyr-433, Asn-439, Gly-444, and Leu-447 substantially arrested NBCe1-A in the ER (data not shown), of which the latter 5 substitutions were reported previously (13). Western blot analysis indicated that the remaining cysteine substitutions were all well expressed and glycosylated similar to NBCe1-A-5C− (data not shown).

Labeling of NBCe1-A-substituted Cysteines with Biotin Maleimide—To determine the intracellular border of NBCe1-A-TM1, we analyzed the aqueous accessibility of the substituted cysteines to a membrane-permeable cysteine-specific reagent, biotin maleimide (BM). BM only labels aqueous-ex-
posed freely reactive cysteines on the surface of a transmembrane protein. If a substituted cysteine is in the lipid bilayer or in an aqueous-inaccessible conformation, it will not be labeled (23, 24). Whole cell labeling of HEK 293 cells expressing NBCe1-A-substituted cysteines with BM was performed after 48 h expression. Fig. 2, upper panel, shows that the negative control, NBCe1-A-5C/H11002, was minimally labeled, whereas the positive control, Cys-1035, was strongly labeled. Of the 64 substituted cysteines, the region of Cys-389–Gln-393 was labeled; the region of Arg-394–Thr-442 was unlabeled; and consistent with our previous study (13), the region of Phe-443–Asp-452 was periodically labeled (Fig. 2, lower panel). The lack of BM labeling of the long stretch of amino acids, Arg-394–Thr-442, was surprising because it consists of 49 residues that are unlikely to be fully embedded in the lipid bilayer due to its unusual long size.

Functional Characterization of NBCe1-A-TM1 Cysteine Substitutions

Transport Assay—The cysteine substitutions between Gln-424 and Asp-452 in NBCe1-A-TM1 were previously reported to affect transport function to various degrees (13). To determine the functional effect of cysteine substitutions in the region of Cys-389–Ile-423, we measured the intracellular pH changes associated with NBCe1-A mediated HCO₃⁻ transport using the pH-sensitive fluorescent probe BCECF. Transfected HEK 293 cells were first equilibrated in a Na⁺-free HEPES solution and then exposed to a Na⁺-free HCO₃⁻-buffered solution. The influx of CO₂ quickly decreased the intracellular pH. When the intracellular pH stabilized, Na⁺ was added (substituted for tetramethylammonium), which activated NBCe1-A, leading to intracellular pH recovery in contrast to vector-transfected cells (Fig. 3A). Fig. 3B shows that cysteine substitutions in the Cys-389–Ile-423 region have little effect on NBCe1-A transport function.

Sensitivity of Substituted Cysteines to MTSEA—3 residues in TM1 were previously determined to line the NBCe1-A ion permeation pathway (13). Importantly, Thr-442 is involved in forming a gate for the entrance of substrate ions. Given the structural importance of TM1 in NBCe1-A ion translocation, we analyzed the sensitivity of the substituted cysteines in the region of Cys-389–Ile-423 to the inhibition of a small sulfhydryl reactive reagent, MTSEA. MTSEA is a membrane-permeable reagent that is able to enter the ion permeation pathway of NBCe1-A either extracellularly or intracellularly. It forms a covalent adduct to a pore-lining cysteine and subsequently sterically blocks ion translocation (25, 26). HEK 293 cells expressing the cysteine-substituted constructs were preincubated with 1 mM MTSEA in the HEPES buffer and subsequently
processed for the functional assay. Fig. 3C shows, with a 1-min incubation, that MTSEA completely blocked the transport function of the positive control, T442C, but had no effect on NBCe1-A-5C as well as all the analyzed substituted cysteines, suggesting that none of the residues in the region of Cys-389–Ile-423 line the NBCe1-A ion permeation pathway.

**Structural Insight into the Link Region between NBCe1-A-TM1 and the Cytoplasmic Domain**

Accessibility of Cysteine Substitutions in Arg-394–Ile-423 to MTS-TAMRA—The long stretch of BM unlabeled region, Arg-394–Thr-442, contains 49 amino acids, of which the C-terminal region between Gln-424 and Thr-442 is highly hydrophobic, whereas the N-terminal region between Arg-394–Ala-410 is more hydrophilic, containing a highly charged stretch, 404KDLKRR409. We reasoned that Gln-424–Thr-442 forms a lipid-embedded transmembrane helix, but the remaining BM unlabeled region either may be embedded in the lipid bilayer or may be in a folded aqueous-inaccessible conformation. Our previous study has shown that MTS-TAMRA, a cysteine-specific chemical with a small reactive group, can reach cysteine residues deeper in the protein complex that BM cannot label (4). To further test whether the region of Arg-394–Ile-423 is accessible to the aqueous medium, we performed MTS-TAMRA labeling on the plasma membranes isolated from HEK 293 cells expressing the mutant NBCe1-A. Fig. 4, A and B, show that the positive labeling control Cys-1035 as well as the aqueous-accessible substitutions L392C and Q393C were labeled, whereas the negative control NBCe1-A-5C had no labeling. Other than Lys-404, Arg-408, and Pro-411, none of the residues in the region of Arg-394–Ile-423 was labeled. Interestingly, the 3 labeled residues form a pattern approximately in a 4-fold periodicity.

Accessibility of Cysteine Substitutions in Arg-394–Ile-423 to BM after Na2CO3 Treatment—The MTS-TAMRA labeling indicated that 3 residues in the region of Arg-394–Ile-423 are aqueous-accessible; however, location of the remaining residues was not resolved. Treatment of the isolated plasma membranes with Na2CO3 was shown to expose Arg-298, a residue folded in the NBCe1-A cytoplasmic domain, to aqueous, but not the residues embedded in the lipid bilayer (cysteines in NBCe1-A-5C-) (10). To distinguish whether the BM unlabeled cysteine substitutions reside in the lipid bilayer or in a folded conformation in the cytosol, we treated the plasma membranes isolated from transfected cells with 100 mM Na2CO3 prior to
FIGURE 3. Functional characterization of NBCe1-A-substituted cysteines. A, representative NBCe1-A transport assay. HEK 293 cells transfected with pcDNA3.1 or NBCe1-A-5C \(^{-}\) were loaded with the pH-sensitive probe BCECF-AM and assayed with a microfluorometer. Cells were initially equilibrated in HEPES-buffered Na\(^+\) free solution. Intracellular pH was then acidified by a CO\(_2\)-HCO\(_3\) \(^{-}\)-buffered Na\(^+\) free solution. Upon the addition of a 140 mM Na\(^+\) containing CO\(_2\)-HCO\(_3\) \(^{-}\)-buffered solution, intracellular pH in NBCe1-A-5C \(^{-}\) transfected cells rapidly recovered. All solutions contained 15 μM EIPA to block endogenous Na\(^+\)/H\(^+\) exchange. B, summary of cysteine-substituted NBCe1-A transport function. Transport data were expressed as a percentage of NBCe1-A-5C \(^{-}\) function. Asterisks mark the uncharacterized constructs due to lack of plasma membrane expression. Error bars represent mean ± S.E. (n = 3–6). C, summary of cysteine-substituted NBCe1-A transport function after MTSEA treatment. Transfected HEK 293 cells were incubated with 1 mM MTSEA for 1 min prior to the transport assay. Transport data were expressed as a percentage of activity in the absence of MTSEA. Asterisks mark the uncharacterized constructs due to lack of plasma membrane expression. Error bars represent mean ± S.E. (n = 3–7).
Transmembrane Segment 1 of NBCe1-A

FIGURE 4. MTS-TAMRA labeling of isolated cell membranes. A, representative data of MTS-TAMRA labeling. Membranes isolated from HEK 293 cells expressing various substituted cysteines were resuspended in PBS (pH 7.5) and incubated with 0.1 mM MTS-TAMRA for 20 min at room temperature. NBCe1-A proteins were immunoprecipitated, resolved on 7.5% SDS-PAGE, and transferred to nonfluorescent PVDF membrane. Labeled samples were detected by a Typhoons Scanner 9410 at excitation wavelength 545 nm and emission wavelength of 590 nm. Images from separate gels were used to display the results.

Effect of Missense Mutation S427L on TM1 Orientation

The aforementioned studies have established that NBCe1-A-TM1 consists of 31 amino acids ranging from Phe-412 to Thr-442. This finding localized the pRTA-causing mutation, Ser-427, to the middle of NBCe1-A-TM1. We hypothesized that the side-chain hydrophobicity and bulkiness of amino acids at this position may play a critical structural and functional role and therefore individually substituted Ser-427 with Val and Ile (hydrophobic side chain), Tyr (large side chain), Pro (rigid side chain), and Cys (nucleophilic side chain) for the functional analysis. Immunocytochemistry showed that all the substitutions are well expressed on the plasma membrane of HEK 293 cells (data not shown); however, Val, Ile, Tyr, and Pro substitutions completely inactivated NBCe1-A ion transport, but not Cys substitution, which retained 50% function of the wild-type NBCe1-A (Fig. 6).

Ser-427 resides adjacent to Ala-428, a residue that lines the NBCe1-A ion permeation pathway (13). On a helical wheel plot of TM1, Ser-427 is 100° apart from Ala-428, which is likely to be embedded in the lipid bilayer or in close contact with other TMs. Additionally, residue Thr-442, which resides at the C-terminal end of NBCe1-A-TM1, is on the same surface with Ala-428 that is 100% sensitive to MTSEA inhibition and accessible to MTS-TAMRA labeling. To explore whether Leu substitution at Ser-427 may affect the helix packing of NBCe1-A that alters the TM1 orientation, we compared the accessibility of T442C with MTS-TAMRA in a WT-NBCe1-A background or in the presence of S427L, S427T, or S427A. Fig. 7 (upper and middle panels) shows that T442C is strongly labeled with MTS-TAMRA in the WT-NBCe1-A background, but not in the presence of S427L. Interestingly, when S427T or S427A (data not shown) is present, T442C becomes highly accessible to MTS-TAMRA labeling. Transport assays indicated that T442C/WT-NBCe1-A, T442C/S427T, and T442C/S427A function normally and that T442C/S427L has no measurable function similar to NBCe1-A-S427L (Fig. 7, lower panel).

In addition to Thr-442, Ala-435 and Ala-428 also line the ion permeation pathway but are in deeper positions inaccessible to MTS-TAMRA. To test whether S427L shifts the TM1 orientation affecting aqueous exposure of these two positions, we analyzed the accessibility of A435C and A428C to [14C]NEM in the presence and absence of S427L. Fig. 8, A and B, shows that the positive control Cys-1035 was labeled, whereas the negative control NBCe1-A-SC had no labeling. A435C was labeled, confirming that it is lining the ion permeation pathway, and when S427L was present, labeling of A435C was dramatically decreased. Previously, A428C was found to be accessible to the smallest MTS reagent, MTSEA (13). However, either in the absence or in the presence of S427L, A428C was not labeled when S427L was present, T442C becomes highly accessible to MTS-TAMRA in the WT-NBCe1-A background, but not in the presence of S427L. Interestingly, when S427T or S427A (data not shown) is present, T442C becomes highly accessible to MTS-TAMRA labeling. Transport assays indicated that T442C/WT-NBCe1-A, T442C/S427T, and T442C/S427A function normally and that T442C/S427L has no measurable function similar to NBCe1-A-S427L (Fig. 7, lower panel).

Our previous analyses have determined that 3 residues, Lys-404, Arg-408, and Pro-411, in the cytoplasmic end of TM1 are aqueous-accessible as demonstrated by MTS-TAMRA labeling. To further test whether S427L affects the folding of the N-terminal end of TM1, we individually introduced G400C, K404C, R408C, and P411C into the NBCe1-A-S427L construct. Fig. 8C shows that double substitutions S427L/G400C, S427L/R408C, and S427L/P411C were not glycosylated, indicating BM labeling. Fig. 5 shows, upon Na2CO3 treatment, that the region of Arg-394–Arg-397 was strongly labeled; Gly-400 was moderately labeled; and 3 residues, Lys-404, Arg-408, and Pro-411, were weakly labeled in an otherwise unlabeled region with a 4-fold periodicity. Taken together, these obtained results indicated that the region of Phe-412–Ile-423 is embedded in the lipid bilayer and that the region of Arg-394–Pro-411 is folded in the cytosol.

FIGURE 5. MTS-TAMRA labeling of isolated cell membranes. A, representative data of MTS-TAMRA labeling. Membranes isolated from HEK 293 cells expressing various substituted cysteines were resuspended in PBS (pH 7.5) and incubated with 0.1 mM MTS-TAMRA for 20 min at room temperature. NBCe1-A proteins were immunoprecipitated, resolved on 7.5% SDS-PAGE, and transferred to nonfluorescent PVDF membrane. Labeled samples were detected by a Typhoon Scanner 9410 at excitation wavelength 545 nm and emission wavelength of 590 nm. Images from separate gels were used to display the results.

Effect of Missense Mutation S427L on TM1 Orientation

The aforementioned studies have established that NBCe1-A-TM1 consists of 31 amino acids ranging from Phe-412 to Thr-442. This finding localized the pRTA-causing mutation, Ser-427, to the middle of NBCe1-A-TM1. We hypothesized that the side-chain hydrophobicity and bulkiness of amino acids at this position may play a critical structural and functional role and therefore individually substituted Ser-427 with Val and Ile (hydrophobic side chain), Tyr (large side chain), Pro (rigid side chain), and Cys (nucleophilic side chain) for the functional analysis. Immunocytochemistry showed that all the substitutions are well expressed on the plasma membrane of HEK 293 cells (data not shown); however, Val, Ile, Tyr, and Pro substitutions completely inactivated NBCe1-A ion transport, but not Cys substitution, which retained 50% function of the wild-type NBCe1-A (Fig. 6).

Ser-427 resides adjacent to Ala-428, a residue that lines the NBCe1-A ion permeation pathway (13). On a helical wheel plot of TM1, Ser-427 is 100° apart from Ala-428, which is likely to be embedded in the lipid bilayer or in close contact with other TMs. Additionally, residue Thr-442, which resides at the C-terminal end of NBCe1-A-TM1, is on the same surface with Ala-428 that is 100% sensitive to MTSEA inhibition and accessible to MTS-TAMRA labeling. To explore whether Leu substitution at Ser-427 may affect the helix packing of NBCe1-A that alters the TM1 orientation, we compared the accessibility of T442C with MTS-TAMRA in a WT-NBCe1-A background or in the presence of S427L, S427T, or S427A. Fig. 7 (upper and middle panels) shows that T442C is strongly labeled with MTS-TAMRA in the WT-NBCe1-A background, but not in the presence of S427L. Interestingly, when S427T or S427A (data not shown) is present, T442C becomes highly accessible to MTS-TAMRA labeling. Transport assays indicated that T442C/WT-NBCe1-A, T442C/S427T, and T442C/S427A function normally and that T442C/S427L has no measurable function similar to NBCe1-A-S427L (Fig. 7, lower panel).

In addition to Thr-442, Ala-435 and Ala-428 also line the ion permeation pathway but are in deeper positions inaccessible to MTS-TAMRA. To test whether S427L shifts the TM1 orientation affecting aqueous exposure of these two positions, we analyzed the accessibility of A435C and A428C to [14C]NEM in the absence and presence of S427L. Fig. 8, A and B, shows that the positive control Cys-1035 was labeled, whereas the negative control NBCe1-A-SC had no labeling. A435C was labeled, confirming that it is lining the ion permeation pathway, and when S427L was present, labeling of A435C was dramatically decreased. Previously, A428C was found to be accessible to the smallest MTS reagent, MTSEA (13). However, either in the absence or in the presence of S427L, A428C was not labeled when S427L was present, T442C becomes highly accessible to MTS-TAMRA in the WT-NBCe1-A background, but not in the presence of S427L. Interestingly, when S427T or S427A (data not shown) is present, T442C becomes highly accessible to MTS-TAMRA labeling. Transport assays indicated that T442C/WT-NBCe1-A, T442C/S427T, and T442C/S427A function normally and that T442C/S427L has no measurable function similar to NBCe1-A-S427L (Fig. 7, lower panel).

Our previous analyses have determined that 3 residues, Lys-404, Arg-408, and Pro-411, in the cytoplasmic end of TM1 are aqueous-accessible as demonstrated by MTS-TAMRA labeling. To further test whether S427L affects the folding of the N-terminal end of TM1, we individually introduced G400C, K404C, R408C, and P411C into the NBCe1-A-S427L construct. Fig. 8C shows that double substitutions S427L/G400C, S427L/R408C, and S427L/P411C were not glycosylated, indicating
their misfolding in the ER, whereas S427L/K404C was glycosylated. Because each of the individual substitutions, G440C, R408C, or P411C, had a minimal effect on NBCe1-A membrane processing, in the presence of S427L, the structure of the N terminus of TM1 must be perturbed, leading to protein misfolding. The labeling of K404C with $[^{14}\text{C}]\text{NEM}$ was significantly blocked when S427L was present (Fig. 8, A and B). In summary, our aforementioned observations strongly suggest that the S427L mutation substantially alters the orientation of NBCe1-A-TM1, thereby disrupting the structure of the NBCe1-A ion permeation pathway.

**Topological Determination of AE1-TM1**

*Construction and Surface Processing of AE1-TM1-substituted Cysteines*—The N-terminal region of NBCe1-A-TM1 was previously modeled based on AE1 (13); however, the validity of this comparison has not been confirmed. Our results suggest that the topology of this region in NBCe1-TM1 is unique and may differ from AE1. Furthermore, the topology of the AE1-TM1 N terminus has not been analyzed experimentally. To precisely compare the topological differences of TM1 between NBCe1-A and AE1, we individually substituted 55 amino acids at the position between Leu-378 and Arg-432 that cover the whole predicted AE1-TM1 in a cysteineless AE1-C/H11002 template where 5 endogenous cysteines were replaced with serines. AE1-C/H11002 processes to the plasma membrane and functions normally (23, 27). To determine whether the membrane-processing property of AE1 is impaired by the substituted cysteines, we performed immunocytochemistry analyses with an antibody (AE12-M...
from Alpha Diagnostics) that detects AE1 on the surface of living cells. The results showed that AE1-C/H11002 is well processed on the plasma membrane, whereas the negative control, R808C (28), could only be detected following membrane permeabilization by methanol, which revealed its intracellular retention. Of the 55 substituted cysteines, none were defective in membrane processing (data not shown).

Labeling of AE1-TM1 Cysteine Substitutions with Biotin Maleimide—48 h after transfection, HEK 293 cells expressing the cysteine-substituted AE1 constructs were collected and subjected to whole cell labeling with BM. Fig. 9A shows that the positive control AE1-Cys-201 (only contains one endogenous cysteine) was strongly labeled, whereas the negative control AE1-C- had no labeling. The region of Leu-378 to Ile-397 was labeled to various degrees except that one residue, Asp-396, was minimally labeled; the region of Thr-398–Thr-422 was not labeled; and the region of Phe-423–Arg-432 was labeled weakly with two unlabeled substitutions: Gly-424 and Gly-425 (Fig. 9B). This finding indicated that the linker region between AE1 cytoplasmic domain and the transmembrane domain is highly accessible to aqueous, and the 9 amino acids whose deletion causes SAO reside in the beginning of AE1-TM1 rather than at the lipid/aqueous interface as thought previously (Fig. 9C) (17).

This result demonstrates that the N-terminal TM1 region in the two HCO3− transporters folds differently.

**DISCUSSION**

The TM1 of NBCe1-A plays an essential role in mediating ion transport by its direct involvement in forming part of the ion permeation pathway (13). The structural importance of NBCe1-A-TM1 is also exemplified by the disease-causing missense mutation, S427L, that dramatically impairs the transport function (6). In the present study, we explored the structural mechanism of S427L in abrogating NBCe1-A transport function by an extensive examination of the topological folding of NBCe1-A-TM1 in the native cellular environment. Our findings demonstrated that NBCe1-A-TM1 is structurally unique from AE1-TM1 and is potentially involved in modulating the transport function of NBCe1-A.

Our results have shown that the topology of NBCe1-A-TM1 differs significantly from what was assumed previously (13). Specifically: 1) NBCe1-A-TM1 contains 31 amino acids ranging from Phe-412–Thr-442; 2) none of the amino acids prior to Ala-428 lines the ion permeation pathway; 3) a cryptic region, Arg-394–Pro-411, links the membrane-embedded N-terminal end of TM1 with the cytoplasmic domain; and 4) in comparison...
inhibited. This may be due to two possibilities: 1) the residues are not part of the ion permeation pathway; 2) the residues reside in a wide open region such that attachment of MTSEA has no impact on the transport function. The latter possibility is unlikely because none of these residues were labeled with biotin maleimide or MTS-TAMRA. The minimal functional effect of cysteine substitutions in this region further supports that the N-terminal half of TM1 is not involved in forming the NBCe1-A ion permeation pathway.

In our new NBCe1-A-TM1 topological model, the missense mutation S427L is located in the middle of the lipid bilayer rather than near the intracellular surface. Substitution of Ser-427 with Thr, Ala, or Cys was shown to retain substantial NBCe1-A transport function (6, 10, 12), unlike the S427L mutation. Thr and Cys are both nucleophilic amino acids in the same group with Ser, and Ala has a much smaller side chain when compared with Ser. This seems to indicate that the hydrophobicity and bulkiness of the amino acid side chain at this position have a profound effect on the local structure involved in helix packing of the NBCe1-A transmembrane domain. Indeed, when Ser-427 was substituted with Val or Ile, both of which are structurally similar to Ser but carry hydrophobic side chains, and with Tyr, which carries a bulky side chain, ion transport was completely blocked, unlike S427C, which remains 50% functional (10), fully supporting the aforementioned hypothesis. Functional inactivation induced by Pro substitution at this position also supported that Ser-427 is involved in TM1 helix packing. Further testing of the structural effect of S427L on NBCe1-A-TM1 revealed that Leu substitution at Ser-427 completely impaired ion transport as well as the aqueous access to T442C, a residue residing at the C-terminal end of TM1, but interestingly, when Thr or Ala was substituted at Ser-427, NBCe1-A remained functional and T442C became aqueous-accessible. Additionally, when S427L is present, the aqueous access to Ala-435, an amino acid residing deeper in the NBCe1-A ion permeation pathway, is significantly blocked, and furthermore, the aqueous access to Lys-404, an amino acid residing at the N-terminal end of TM1 is also significantly blocked. These 3 residues (Thr-442, Ala-435, and Lys-404) are distributed throughout the length of TM1, suggesting that S427L triggers an overall conformational change of TM1. Taken together, our findings indicate that Ser-427 is located in a space-confined region with its nucleophilic side chain involved in TM1 helix packing with other TMs in the NBCe1-A transmembrane domain. Leu substitution at this position abolishes the potential ionic interaction and subsequently alters the orientation of TM1. This alteration is expected to change the configuration and/or collapse the NBCe1-A ion permeation pathway and thereby inactivate ion transport.

The lack of BM labeling in the linker region between NBCe1-A-TM1 and the cytoplasmic domain is in a folded conformation that may be involved in protein-protein interactions. The longer than expected NBCe1-A-TM1 indicates that it must adopt a tilted position in the plasma membranes to accommodate its lipid embedding. The residues (Asp-416, Gln-424, Tyr-433, and Asn-439) whose substitutions misfold NBCe1-A, triggering ER retention, are distributed throughout the TM1 helix (Fig. 10A), and furthermore, in the helical wheel plot, they are located on different surfaces of the helix (Fig. 10B). This suggests that TM1 may have a rigid position within NBCe1-A protein complex and that substitution of the residues responsible for maintaining TM1 in its native position results in NBCe1-A misfolding. The C-terminal extracellular half of TM1 is involved in forming part of the NBCe1-A ion permeation pathway (13); however, after screening 13 cysteine substitutions in the N-terminal half of TM1 (Pro-411–Ile-423) with MTSEA, we determined that none of the substitutions were functionally involved in forming part of the NBCe1-A ion permeation pathway.
(D405C, K409C, and D416C) that lead to NBCe1-A misfolding are clustered on a surface within 40° apart from the above residues (Fig. 10B). This unique pattern of amino acid grouping in the helical wheel plot strongly suggests that Arg-394–Pro-411 forms a helical structure. We propose that the surface clustered with Lys-404, Arg-408, and Pro-411 is in a folded cytoplasmic region with minimal access to aqueous and that the surface clustered with Asp-405, Lys-409, and Asp-416 partakes in hel-
flexible (20). Our present data obtained in situ fully support this finding, and furthermore, precisely determined that the C-terminal end of the flexible linker is at Ile-397. Our data demonstrate that the 9 amino acids (Ala-400–Ala-408) in the linker region whose deletion causes SAO (17, 18) reside in the lipid bilayer forming the N-terminal end of AE1-TM1. These results indicate that the topology of NBCe1-A-TM1 differs significantly from AE1-TM1.

In summary, we have shown for the first time that NBCe1-A-TM1 contains 31 membrane-embedded amino acids and that Ser-427 resides in the middle of NBCe1-A-TM1, whose Leu substitution alters the orientation of TM1, impairing NBCe1-A ion transport. Our results are compatible with the prediction that NBCe1-A-TM1 adopts a tilted position in the transmembrane region with its cytosolic N-terminal portion Arg-394–Pro-411 (in helical conformation) in tight interaction with the cytoplasmic domain. This raises the interesting question as to whether the N-terminal cytoplasmic domain of NBCe1-A is involved in mediating or modulating NBCe1-A ion transport.

REFERENCES

1. Pushkin, A., and Kurtz, I. (2006) SLC4 base (HCO3−/CO2−) transporters: classification, function, structure, genetic diseases, and knockout models. Am. J. Physiol. Renal Physiol. 290, F580–F599
2. Boron, W. F. (2006) Acid-base transport by the renal proximal tubule. J. Am. Soc. Nephrol. 17, 2368–2382
3. Kao, L., Sassani, P., Azimov, R., Pushkin, A., Abuladze, N., Peti-Peterdi, J., Liu, W., Newman, D., and Kurtz, I. (2008) Oligomeric structure and minimal functional unit of the electrogenic sodium bicarbonate cotransporter NBCe1-A. J. Biol. Chem. 283, 26782–26794
4. Zhu, Q., Kao, L., Azimov, R., Abuladze, N., Newman, D., Pushkin, A., Liu, W., Chang, C., and Kurtz, I. (2010) Structural and functional characterization of the C-terminal transmembrane region of NBCe1-A. J. Biol. Chem. 285, 37178–37187
5. Igashiri, T., Inatomi, J., Sekine, T., Cha, S. H., Kanai, Y., Kunimi, M., Tsukamoto, K., Satoh, H., Shimadzu, M., Tozawa, F., Mori, T., Shiobara, M., Seki, G., and Endou, H. (1999) Mutations in SLC4A4 cause permanent isolated renal tubular acidosis with ocular abnormalities. Nat. Genet. 23, 264–266
6. Dinour, D., Chang, M. H., Satoh, J., Smith, B. L., Angle, N., Knecht, A., Serban, I., Holtzman, E. J., and Romero, M. F. (2004) A novel missense mutation in the sodium bicarbonate cotransporter (NBCe1/SLC4A4) causes proximal tubular acidosis and glaucoma through ion transport defects. J. Biol. Chem. 279, 52238–52246
7. Horita, S., Yamada, H., Inatomi, J., Moriyama, N., Sekine, T., Igashiri, T., Endo, Y., Dasouki, M., Ekim, M., Al-Gazali, L., Shimadzu, M., Seki, G., and Fujita, T. (2005) Functional analysis of NBC1 mutants associated with proximal renal tubular acidosis and ocular abnormalities. J. Am. Soc. Nephrol. 16, 2270–2278
8. Suzuki, M., Vaisbich, M. H., Yamada, H., Horita, S., Li, Y., Sekine, T., Moriyama, N., Igashiri, T., Endo, Y., Cardoso, T. P., de Sá, L. C., Koch, V. H., Seki, G., and Fujita, T. (2008) Functional analysis of a novel missense NBC1 mutation and of other mutations causing proximal renal tubular acidosis. Pflugers Arch. 455, 583–593
9. Lo, Y. F., Yang, S. S., Seki, G., Yamada, H., Horita, S., Yamazaki, O., Fujita, T., Usui, T., Tsai, J. D., Yu, I. S., Lin, S. W., and Lin, S. H. (2011) Severe metabolic acidosis causes early lethality in NBC1 W516X knock-in mice as a model of human isolated proximal renal tubular acidosis. Kidney Int. 79, 730–741
10. Zhu, Q., Kao, L., Azimov, R., Newman, D., Liu, W., Pushkin, A., Abuladze, N., and Kurtz, I. (2010) Topological location and structural importance of the NBCe1-A residues mutated in proximal renal tubular acidosis. J. Biol. Chem. 285, 13416–13426
Transmembrane Segment 1 of NBCe1-A

11. Li, H. C., Szüögeti, P., Worrell, R. T., Matthews, J. B., Conforti, L., and Soleimani, M. (2005) Missense mutations in Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter NBC1 show abnormal trafficking in polarized kidney cells: a basis of proximal renal tubular acidosis. *Am. J. Physiol. Renal Physiol.* **289**, F61–F71

12. Yang, H. S., Kim, E., Lee, S., Park, H. J., Cooper, D. S., Rajbhandari, L., and Choi, I. (2009) Mutation of aspartate 555 of the sodium/bicarbonate transporter SLC4A4/NBCe1 induces chloride transport. *J. Biol. Chem.* **284**, 15970–15979

13. Zhu, Q., Azimov, R., Kao, L., Newman, D., Liu, W., Abuladze, N., Pushkin, A., and Kurtz, I. (2009) NBCe1-A transmembrane segment 1 lines the ion translocation pathway. *Biochem. J.* **390**, 137–144

14. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na\(^{+}\)/Cl\(^{-}\)-dependent neurotransmitter transporters. *Nature* **437**, 215–223

15. Watanabe, A., Choe, S., Chaptal, V., Rosenberg, J. M., Wright, E. M., Grabe, M., and Abramson, J. (2010) The mechanism of sodium and substrate release from the binding pocket of vSGLT. *Nature* **468**, 988–991

16. Jarolim, P., Palek, J., Hassan, K., Sapak, P., Nurse, G. T., Rubin, H. L., Zhai, S., Sahr, K. E., and Liu, S. C. (1991) Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11022–11026

17. Mohandas, N., Winardi, R., Knowles, D., Leung, A., Parra, M., George, E., Conboy, J., and Chasis, J. (1992) Molecular basis for membrane rigidity of hereditary ovalocytosis. A novel mechanism involving the cytoplasmic domain of band 3. *J. Clin. Invest.* **89**, 686–692

18. Steck, T. L., Ramos, B., and Strapazon, E. (1976) Proteolytic dissection of band 3, the predominant transmembrane polypeptide of the human erythrocyte membrane. *Biochemistry* **15**, 1153–1161

19. Zhang, D., Kiyatkin, A., Bolin, J. T., and Low, P. S. (2000) Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. *Blood* **96**, 2925–2933

20. Bok, D., Schiöler, M. J., Pushkin, A., Sassani, P., Abuladze, N., Nasser, Z., and Kurtz, I. (2001) Immunolocalization of electrogenic sodium-bicarbonate cotransporters pNBC1 and kNBC1 in the rat eye. *Am. J. Physiol. Renal Physiol.* **281**, F920–F935

21. Guan, L., and Kaback, H. R. (2007) Site-directed alkylation of cysteine to test solvent accessibility of membrane proteins. *Nat. Protoc.* **2**, 2012–2017

22. Zhu, Q., Lee, D. W., and Casey, J. R. (2003) Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1. *J. Biol. Chem.* **278**, 3112–3120

23. Zhu, Q., and Casey, J. R. (2007) Topology of transmembrane proteins by scanning cysteine accessibility mutagenesis methodology. *Methods* **41**, 439–450

24. Karlin, A., and Akabas, M. H. (1998) Substituted-cysteine accessibility method. *Methods Enzymol.* **293**, 123–145

25. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science* **258**, 307–310

26. Zhu, Q., and Casey, J. R. (2004) The substrate anion selectivity filter in the human erythrocyte CI\(^{-}\)/HCO\(_3\)\(^{-}\) exchange protein, AE1. *J. Biol. Chem.* **279**, 23565–23573

27. Quilty, J. A., and Reithmeier, R. A. (2000) Trafficking and folding defects in hereditary spherocytosis mutants of the human red cell anion exchanger. *Traffic* **1**, 987–998