A Substrate Access Tunnel in the Cytosolic Domain Is Not an Essential Feature of the Solute Carrier 4 (SLC4) Family of Bicarbonate Transporters

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Background: A mutation (R298S) in NBCe1 induces a transport defect and causes proximal renal tubular acidosis.

Results: The equivalent mutation (R283S) in AE1 disrupted an H-bonding network without affecting functional expression.

Conclusion: Arg283 stabilizes the cytosolic domain but is not essential for transport.

Significance: A substrate tunnel in the cytosolic domain is not an essential feature of the SLC4 family of bicarbonate transporters.

Anion exchanger 1 (AE1; Band 3; SLC4A1) is the founding member of the solute carrier 4 (SLC4) family of bicarbonate transporters that includes chloride/bicarbonate AEs and Na+-bicarbonate co-transporters (NBCs). These membrane proteins consist of an amino-terminal cytosolic domain involved in protein interactions and a carboxyl-terminal membrane domain that carries out the transport function. Mutation of a conserved arginine residue (R298S) in the cytosolic domain of NBCe1 (SLC4A4) is linked to proximal renal tubular acidosis and results in impaired transport function, suggesting that the cytosolic domain plays a role in substrate permeation. Introduction of single and double mutations at the equivalent arginine (Arg283) and at an interacting glutamate (Glu85) in the cytosolic domain of human AE1 (cdAE1) had no effect on the cell surface expression or the transport activity of AE1 expressed in HEK-293 cells. In addition, the membrane domain of AE1 (mdAE1) efficiently mediated anion transport. A 2.1-Å resolution crystal structure of cdΔ54AE1 (residues 55–356 of cdAE1) lacking the amino-terminal and carboxyl-terminal disordered regions, produced at physiological pH, revealed an extensive hydrogen-bonded network involving Arg283 and Glu85. Mutations at these residues affected the pH-dependent conformational changes and stability of cdΔ54AE1. As these structural alterations did not impair functional expression of AE1, the cytosolic and membrane domains operate independently. A substrate access tunnel within the cytosolic domain is not present in AE1 and therefore is not an essential feature of the SLC4 family of bicarbonate transporters.

The SLC4 genes encode a family of bicarbonate transporters that operate as anion exchangers (AEs) or Na+-bicarbonate co-transporters (NBCs) (1–4). These transporters play an essential role in regulating intracellular pH (pHi) and in the transport of bicarbonate across biological membranes. Mutations in these genes are linked to a variety of human diseases that affect red cell morphology and acid-base homeostasis in other cells (4, 5). For example, mutations in the SLC4A1 gene that encodes AE1 (Band 3) of human red cells cause hereditary spherocytosis due to a deficiency in the complement of AE1 in the mature erythrocyte (6). The AE1 gene also encodes a truncated form of AE1 missing the first 65 residues that is expressed in the α-intercalated cells of the renal collecting duct (kAE1) responsible for bicarbonate reabsorption into the blood and facilitating acid secretion into the urine (7, 8). Mutations in AE1 can cause distal renal tubular acidosis, resulting in impaired acid secretion and metabolic acidosis (4, 9, 10). Mutations in the membrane domain of AE1 (mdAE1) that cause hereditary spherocytosis (11) or distal renal tubular acidosis (10, 12–14) often induce folding defects, resulting in impaired trafficking of the protein from its site of synthesis in the endoplasmic reticulum (ER) to the cell surface. Some dominant distal renal tubular acidosis mutants can be rescued to the cell surface by interfering with the interaction of kAE1 with the ER lectin chaperone calnexin that is involved in ER protein quality control (15, 16). Mutations in the cytosolic domain of AE1 (cdAE1) can also cause hereditary spherocytosis, but these mutations affect the ability of this domain to interact with cytoskeletal proteins, thus interfering with the transport function. This article contains supplemental Figs. 1–3 and Table 1.

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The abbreviations used are: SLC, solute carrier; AE, anion exchanger; cdAE1, cytosolic domain of AE1; cdΔ54AE1, cytosolic domain of AE1 missing the first 54 residues; ER, endoplasmic reticulum; mdAE1, membrane domain of AE1; NBC, Na+-bicarbonate co-transporter; NBCe, electrogenic Na+/HCO3− co-transporter; pHi, intracellular pH; kAE1, kidney AE1; cdkAE1, cytosolic domain of kidney AE1; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol.

This article contains supplemental Figs. 1–3 and Table 1.
such as protein 4.2, without profound effects on the structure of the domain or the ability of AE1 to traffic to the plasma membrane (17, 18).

Mutations in the gene encoding SLC4A4 (NBCe1), a protein expressed in the basolateral surface of the proximal tubule of the kidney, can give rise to proximal renal tubular acidosis and defective acid secretion by the kidney (2, 19). One of these mutations, R298S (human sequence), causes defective bicarbonate absorption across the basolateral membrane and metabolic acidosis due to an impairment in its functional expression (20–22). When expressed in Xenopus oocytes, NBCe1 R298S exhibited reduced transport activity without a major effect on cell surface expression (23). Arg298 in NBCe1 is highly conserved across the SLC4 family of transporters (see Fig. 1); the equivalent residue in human AE1 is Arg283. Structural modeling of the cytosolic domain of NBCe1 using the crystal structure of the human cdAE1 (24) predicted that Arg298 is located in a solvent-inaccessible polar pocket and interacts with Glu81 (Glu85 in human AE1) (23). The R298E mutation had reduced transport activity similar to the natural R298S mutant, but the E91R mutant had a more severe ion transport defect. Interestingly, the double charge reversal mutant (R298E/E91R) had normal transport activity (23). Thus, perturbation of this ionic interaction by site-directed mutagenesis resulted in reduced transport activity, prompting the suggestion that the cytosolic domain of NBCe1 contains a tunnel made up of a chain of interconnected polar residues that creates an ion transport pathway leading substrate to the membrane domain (23).

The cdAE1 is not known to play an essential role in anion transport as its removal by proteolysis does not affect the transport activity of AE1 (25). However, it is conceivable that the cdAE1 like NBCe1 may contain a substrate access tunnel or that this domain may regulate access to the transport pathway in mdAE1. The cdAE1 undergoes dramatic conformational changes with pH (26, 27), providing a possible mechanism to regulate anion transport by opening and closing the tunnel via local changes in intracellular pH (pHi) (28). Removing the cytosolic domain would not be expected to impair transport; however, closing the tunnel through a regulated process or by a mutation as proposed for R298S in NBCe1 could conceivably affect transport by compromising substrate access to the membrane domain.

To test for the presence of a substrate access tunnel in human AE1, we made a series of mutations in AE1 at sites homologous to Arg298 (Arg283 in AE1) and Glu91 (Glu85 in AE1) in NBCe1 and examined their effect on the expression, localization, and transport function of AE1 in transfected HEK-293 cells. Trafficking or functional defects were not found in these mutants, suggesting that human AE1 does not contain an essential substrate access tunnel in its cytosolic domain. Furthermore, by deleting the disordered acidic N-terminal and C-terminal linker regions, we were able to crystallize cdS4AE1 (residues 55–356) at neutral pH and to solve the structure to high resolution (2.1 Å), confirming the presence of a hydrogen-bonded network at physiological pH involving Arg283, Glu85, and neighboring residues contained within a polar pocket. Mutations in these residues affected the sensitivity of cdAE1 to structural changes induced by pH and by urea denaturation. Hydrodynamic studies showed that the domain with the R283S mutation was more asymmetric and prone to aggregation. Furthermore, it could not be crystallized under conditions similar to the wild-type protein, consistent with a disturbed structure. Although mutation of Arg283 affected the structure and stability of the cytosolic domain, alterations at this site did not impair the transport function of AE1, highlighting the structural and functional independence of the cytosolic and membrane domains. We conclude that a substrate access tunnel is not present in the cytosolic domain of AE1 and is therefore not an essential feature of the SLC4 family of bicarbonate transporters.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The entire coding sequence for human AE1 was inserted into the Xhol and BamHI sites of pcDNA3, and an HA tag was inserted into the third extracellular loop to facilitate immunodetection as described previously (29). Mutations were made using a QuikChange™ mutagenesis kit (Agilent Technologies, Santa Clara, CA) using complementary mutagenic primers. DNA sequencing of all constructs was performed by ACGT Corp. (Toronto, Canada) to confirm the presence of the introduced point mutations.

**Cell Transfection**—HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) calf serum, 0.5% penicillin, and 0.5% streptomycin under 5% CO2 at 37 °C as described previously (11). Cells were transfected using either the calcium phosphate method with 1 μg of cDNA/well of a 6-well plate or by Lipofectamine™ 2000 (Invitrogen) to better conserve protein expression. HEK-293 cells were transiently transfected with plasmids containing mutated or wild-type AE1 cDNA and grown on glass coverslips as described previously (11). Cells were fixed with 3.8% formaldehyde. A 1:1,000 dilution of mouse monoclonal anti-HA antibody (Covance, Princeton, NJ). Expression levels were normalized for protein loading to the band intensity of endogenous GAPDH using a mouse monoclonal anti-GAPDH antibody.

**Immunofluorescence**—For immunofluorescence staining, HEK-293 cells were transfected with various AE1 constructs and grown on glass coverslips as described previously (11). Cells were fixed with 3.8% formaldehyde. A 1:1,000 dilution of mouse monoclonal anti-HA antibody followed by a 1:1,000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG antibody was used to detect HA-tagged AE1 in non-permeabilized cells. Washed cells were then permeabilized with 0.2% Triton X-100 and incubated with mouse anti-HA antibody followed by antimouse IgG-Cy3 to detect total AE1 expression as described previously (30). Samples were examined using a Zeiss LSM-510 laser confocal microscope.

**Anion Exchange Activity Assays**—HEK-293 cells were grown on coverslips coated with poly-l-lysine in 100-mm dishes. Cells were transiently transfected with plasmids containing mutated or wild-type AE1 cDNA. Anion exchange assays were performed as described previously (31, 32). Briefly, 2 days post-transfection, coverslips were rinsed with serum-free DMEM and placed in serum-free DMEM containing 2 μM 2′,7′-bis(2-
carboxyethyl)-5-(and -6-)carboxyfluorescein acetoxyethyl ester. After incubation at 37 °C for 15 min, coverslips were mounted in a cuvette and alternately perfused (3.5 ml/min) with Ringer’s buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO_4, 2.5 mM NaH_2PO_4, 25 mM NaHCO_3, 10 mM HEPES, pH 7.4) containing either 140 mM NaCl or 140 mM sodium gluconate at 20 °C. Buffers were continuously bubbled with air containing 5% CO_2 during the experiment. Intracellular fluorescence changes were monitored in a Photon Technologies International RCR fluorometer (London, Ontario, Canada) at excitation wavelengths of 440 and 503 nm and an emission wavelength of 529 nm. pH_i was obtained from fluorescence data by calibration using the nigericin/high potassium method (33). Initial rates of pH_i changes during the first 30 s following change of buffer were measured by linear regression to provide a measure of anion exchange activity. Each mutant was assayed in at least three different transfected preparations. The initial rates were corrected for the low level of background activity seen in cells transfected with the empty vector control. Transport activity was normalized to the total expression of AE1 as quantified on immunoblots probed with IVF-12 mouse anti-AE1 monoclonal antibody (34).

Production and Characterization of cdAE1—The cdAE1 (residues 1–356), the cytosolic domain of AE1 missing the first 54 residues (cdΔ54AE1; residues 55–356), the cytosolic domain of kidney AE1 (cdkAE1; residues 65–356), the mdAE1 (residues 361–911), and various tunnel mutants were expressed as C-terminal His_6-tagged constructs in Escherichia coli and purified by nickel affinity and gel filtration chromatography as described previously (35). The secondary structure of cdAE1 and cdΔ54AE1 was determined using circular dichroism, and their sensitivity to pH and urea was studied using intrinsic fluorescence also as described previously (35). Light scattering measurement were made using a Malvern Viscotec TDAmax low angle light scattering instrument connected to a Superdex S200 10/300 column.

Crystallization of cdΔ54AE1—The cytosolic domain of wild type and tunnel mutants of cdAE1 lacking the disordered N- and C-terminal regions (cdΔ54Δ1; residues 55–356) were expressed and purified as follows. The pET-Blue 1 plasmid containing the coding sequence of human cdΔ54AE1; residues 55–356) was transformed into E. coli BL21(DE3) pLacI Tuner cells. An overnight culture was grown in the presence of 50 mg/ml carbenicillin and 34 mg/ml chloramphenicol in Luria-Bertani (LB) broth medium. After incubation, the culture was diluted 1:100 in 1 liter of yeast extract-tryphtone broth supplemented with antibiotic and grown at 37 °C until the A_600 reached 0.8–1. Protein expression was induced by 0.2 mM isopropyl-1-thio-β-D-galactopyranoside, and the bacteria were further grown for 16 h at 22 °C. Bacterial cells were harvested by centrifugation at 4,000 rpm for 25 min (Sorvall RC-38, H600A rotor) at 4 °C, and pellets were resuspended 1:3 mass (~10 g (wet weight) of cell paste) per volume in lysis buffer (300 mM NaCl, 20 mM imidazole, 20 mM Tris-HCl, pH 8.0 containing 1 mM PMSF), frozen in liquid nitrogen, and stored at −80 °C.

For protein purification, the cell suspensions from three 1-liter batches of cells were thawed in 0 °C chilled water and then disrupted by two passes through an SLM Aminco French press. Insoluble material was removed by ultracentrifugation at 100,000 × g (Beckman L8-70M, T770 rotor) at 4 °C. For purification, the supernatant (~100 ml) was applied to a 5-ml nickel-nitritolriacetic acid column (HisTrap, GE Healthcare) connected to ÄKTA Prime (GE Healthcare). The column was washed with 400 ml of lysis buffer at 0.5 ml/min, and then the His-tagged protein was eluted with 250 mM imidazole buffer. Fractions containing the highest protein concentration were combined (~5 ml) and loaded onto a Superdex S200 16/600 column (GE Healthcare) pre-equilibrated with the gel filtration buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.5). Fractions containing the cdAE1 protein were pooled and concentrated by an Amicon Ultracel 10,000 centrifugal filter to >10 mg/ml at 4,000 rpm (Beckman Allegra X-22R). Protein concentration in a diluted sample was estimated by the DC Protein Assay according to the manufacturer’s protocol (Bio-Rad). Purity of the protein preparations was assessed by SDS-polyacrylamide gel electrophoresis.

Protein Crystallization—For crystallization trials, protein samples were diluted to 5 mg/ml with gel filtration buffer. The initial screens were made by the sitting drop method using commercial screening kits: MCSG1-4 (Microlytic), Index HT (Hampton), and Crystal Screen I (Hampton). Rod-shaped crystal clusters appeared after 1 month of incubation at 22 °C under various conditions; all contained PEG 2000–4000 with pH values ranging from 6.5 to 7.0. Because of higher reproducibility, PEG 3350 was selected for screen refinement. After fine screening, prismatic rod-shaped crystals (0.1–0.3 mm in length) were obtained using a microseeding technique in conditions containing 100 mM BisTris, pH 6.5, 18% PEG 3350 with 0.5% (w/v) octyl glucoside as an additive.

Data Collection and Structure Determination—Crystals were soaked in cryoprotectant (25% PEG 3350, 5% glycerol, 100 mM BisTris, pH 6.5) for 2 min prior to being flash frozen in liquid nitrogen. Crystallographic data were collected on a single crystal at 105 K on Northeastern Collaborative Access Team beam line 24-ID-E at the Advanced Photon Source (Chicago, IL). Diffraction data for cdΔ54AE1 were collected at wavelength 0.9795 using 100 images with 1° oscillations. Data were processed with XDS to a resolution of 2.1 Å. The first structural model was obtained by molecular replacement using a single chain of the published structure of cdAE1 (Protein Data Bank code 1HYN) (24) as a search model in the program Phaser (36, 37), which yielded a log likelihood gain of 3015. The final model was generated following several rounds of model building using the molecular graphic program Coot (38) and refinement using PHENIX REFINEx (39), enabling non-crystallographic symmetry (NCS) and translation/liberation/screw (TLS) (15 groups) (40), yielding a final R_work/R_free of 18.5/22.3. No electron density was visualized for residues 55–56, 181–183, 203–217, and 351–356 in chain A and 55, 203–217, and 349–356 in chain P or for the C-terminal His_6 tag and linker. There were no outliers in the Ramachandran plot with 95.4% in the favored region and 4.6% in the allowed region. Please see supplemental Table 1, a crystallography table of statistics, for full details. The atomic coordinates and structure factors for cdΔ54AE1 (code 4KY9) have been deposited in the Protein Data Bank, Research Col-

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Expression of AE1 Mutants in HEK-293 Cells—A panel of single and double tunnel mutants at equivalent positions (Arg<sup>283</sup> and Glu<sup>85</sup>; Fig. 1) to those (Arg<sup>298</sup> and Glu<sup>91</sup>) conserved in human NBCe1 (Fig. 1) were prepared in human AE1 and expressed in HEK-293 cells. An HA tag was engineered into the cytosolic domain (residues 66–356); mdAE1 (residues 361–911) that lacks the entire cytosolic domain; and two additional mem- 
brane domain (E681Q and P868L) were similar to AE1 (Fig. 2, lane 11) versus lanes 1–7 and 12

Immunolocalization of AE1 Mutants—We next examined the cellular localization of the tunnel mutants using immunofluorescence staining and confocal microscopy to determine whether the mutation affected trafficking of AE1 to the cell surface (Fig. 3). AE1 HA-tagged in extracellular loop 3 was localized at the cell surface in intact cells (green). Cells were then permeabilized, and the total population of AE1 was detected (red). AE1 showed a predominant cell surface staining (yellow corresponds to overlap of green and red) in transfected HEK-293 cells as observed previously (11) as did all the tunnel mutants and mdAE1. AE1 and the tunnel mutants also showed intracellular staining (red) that co-localized with calnexin, an ER marker (not shown). Similarly, kAE1 and the E681Q and P868L mutants also showed cell surface expression, whereas the AE1SAO deletion mutant was entirely localized intracellularly in the ER as reported previously (46). Thus, introducing the tunnel mutations into the cytosolic domain of AE1 does not prevent their cell surface expression. Indeed, the mdAE1 was also localized at the cell surface, showing that the cytosolic domain is not required for the trafficking of AE1 to the plasma membrane.

Bicarbonate Transport Activity of AE1 Mutants—The anion transport activities of the tunnel mutants were tested in transfected HEK-293 cells loaded with 2′,7′-bis(2-carboxyethyl)-5-
and -6-)carboxyfluorescein, a pH-sensitive fluorescent dye (Fig. 4). Cells equilibrated in bicarbonate- and chloride-containing medium were shifted to chloride-free medium, which induced AE1-mediated chloride exit from the cells in exchange for bicarbonate, leading to alkalinization of the cytosol. Readition of chloride-containing medium led to bicarbonate efflux in exchange for chloride and acidification of the cytosol (32, 47). HEK-293 cells transfected with empty vector (pcDNA3) had little detectable chloride/bicarbonate exchange activity, whereas wild-type (WT) AE1 had robust activity (Fig. 4A). The

TABLE 1

Functional expression of HA-tagged AE1 mutants in HEK-293 cells

The HA tag was inserted into the third extracellular loop of AE1 to facilitate detection on immunoblots and at the cell surface of transfected intact HEK-293 cells. ND, not determined; pRTA, proximal renal tubular acidosis.

| Construct      | Relative expression levels | Cell surface expression* (+/−) | Cell surface expression* | Relative transport activity* | Comments                      |
|----------------|---------------------------|--------------------------------|--------------------------|------------------------------|-------------------------------|
| AE1            | (100)                     |                               |                          |                              | HA-tagged control              |
| E85A           | 97 ± 12                   | +                              | 42 ± 5                   | (100)                        | Arg<sup>283</sup>-interacting residue |
| E85R           | 111 ± 34                  | +                              | 50 ± 3                   | 114 ± 12                     | Charge reversal                |
| R283A          | 113 ± 24                  | +                              | 31 ± 2                   | 114 ± 1                      | Glu<sup>34</sup>-interacting residue |
| R283E          | 130 ± 37                  | +                              | 49 ± 1                   | 141 ± 15                     | Charge reversal                |
| R283S          | 129 ± 13                  | +                              | 42 ± 3                   | 120 ± 16                     | pRTA equivalent mutant         |
| E85R/R283E     | 109 ± 16                  | +                              | 57 ± 1                   | 131 ± 12                     | Double reversal mutant         |
| kAE1           | 163 ± 20                  | +                              | ND                       | ND                           | Truncated cytosolic domain     |
| mdAE1          | 88 ± 13                   | +                              | 47 ± 2                   | 118 ± 14                     | No cytosolic domain            |
| E681Q          | 84 ± 28                   | +                              | 40 ± 4                   | 18 ± 3                       | Low transport mutant           |
| P868L          | 114 ± 18                  | +                              | 38 ± 1                   | 111 ± 11                     | High transport mutant          |
| AE1SAO         | ND                        | −                              | ND                       | ND                           | Inactive, retained in ER       |
| N642D-His      | 97 ± 6                    | +                              | 40 ± 1                   | 123 ± 13                     | N-Glycosylation mutant         |
| AE1 (untagged) | ND                        | +                              |                          |                              | No HA tag                      |

* Determined by immunofluorescence detection of HA tag in intact cells.
* Determined by cell surface biotinylation.
* Corrected for background activity of the vector control and normalized for the amount of AE1 at the cell surface.
**FIGURE 3. Localization of human AE1 mutants in transfected HEK-293 cells using immunofluorescence and confocal microscopy.** All constructs had an HA epitope inserted in the third extracellular loop to allow detection of cell surface expression in intact cells. Fixed and non-permeabilized cells were incubated with mouse anti-HA antibody followed by Alexa Fluor 488-conjugated anti-mouse antibody (green) to detect cell surface AE1. Washed cells were then permeabilized with detergent and incubated with mouse anti-HA antibody followed by anti-mouse Cy3 (red) to detect total AE1 expression. Yellow is the overlap of the green and red and represents AE1 found at the cell surface. Calnexin, a marker for the ER, was detected using a rabbit anti-calnexin antibody followed by anti-rabbit IgG-Cy5 (not shown). Note that all mutants except AE1SAO, which is found exclusively in the ER, were present at the cell surface (yellow) as well as in the ER (red).

R283S mutant had a transport activity similar to wild-type AE1. The E681Q mutant was impaired in transport but had residual activity above the vector control, showing that it was not completely inactive as determined by this assay in HEK cells.

The results of three independent transport assays of the panel of mutants corrected for background transport activity of the vector control and normalized to cell surface expression levels as determined by biotinylation assays are shown in Fig. 4B and are summarized in Table 1. All of the tunnel mutants showed cell surface expression levels and whole cell anion transport activities comparable with wild-type AE1 (Table 1). AE1 R283S, containing the mutation at the homologous position that causes proximal renal tubular acidosis mutation in NBCe1, had transport activity similar to AE1. Thus, this arginine residue does not play an essential role in the anion translocation process by AE1. The E85A and E85R mutants also showed normal transport activity, suggesting that this residue is also not essential for the transport process in AE1. The double “charge reversal” mutant (E85R/R283E) showed normal transport activity too. Expression of the membrane domain of AE1 in HEK-293 cells resulted in a high level of transport activity, confirming that the cytosolic domain is not required for transport activity (25). AE1 with an external HA tag was used as a control as the tunnel mutants also contained the HA tag and were derived from this construct. The HA-tagged AE1 had similar transport activity compared with untagged AE1 in two different vectors, pcDNA3 (AE1WT) (11) and pJRC9 (JRAE1) (47) (Table 1). Therefore, the introduction of the extracellular HA tag into extracellular loop 3 does not impair the functional expression of AE1.

The transport assay data show that none of the tunnel mutants were impaired in transport activity to the level of the E681Q mutant (Table 1). Glu681 was shown previously to be required for chloride/bicarbonate exchange (42, 43, 48, 49) and was used as a transport-defective control. The E681Q mutant had a very low transport activity (18% versus AE1-HA) in transfected cells but slightly above the empty vector control, indicating some residual transport activity. We also tested P868L, which is reported to have a 2–3-fold enhanced transport activity in red blood cells (45). This mutant, however, had a normal level of transport activity when assayed in transfected HEK-293 cells. This suggests that the transport activity of this mutant is dependent upon its cellular context. HEK-293 cells, for example, do not express red cell proteins, including Glycophorin A, that are known to influence the transport activity of AE1 (50–52). The transport assays clearly show that mutation of residue Arg283 or Glu85 within the cytosolic domain does not impair the anion transport activity of AE1 when expressed in HEK-293 cells.
Crystal Structure of cd\(\Delta 54AE1\)—In 2000, a crystal structure of cd\(\Delta 54AE1\) obtained to 2.6-Å resolution was reported by Low and co-workers (24). It revealed the dimeric nature of cd\(\Delta 54AE1\) and many of its important structural features. The crystals were produced from recombinant protein at low pH (4.8) using ammonium sulfate as the precipitant (53, 54). The cytosolic domain of AE1 undergoes dramatic structural changes going from a compact structure at low pH to a more open and asymmetric structure at neutral and alkaline pH (26, 27, 53, 55), leaving open the question of whether the structure obtained under acidic conditions differs from the structure at neutral pH. To obtain a crystal structure of cd\(\Delta 54AE1\) under more physiological conditions, we removed the intrinsically disordered acidic N-terminal region (residues 1–54) and the C-terminal linker region (residues 357–379) not visible in the original crystal structure. We were able to produce a new crystal form of cd\(\Delta 54AE1\) (residues 55–356) using PEG as precipitant at pH 6.5 that diffracted to 2.1 Å (Fig. 5 and supplemental Table 1). The space group was P2\(_1\)2\(_1\)2\(_1\) with two monomers in the asymmetric unit (\(a\), 69.9 Å; \(b\), 80.7 Å; \(c\), 104.3 Å with \(\alpha\), \(\beta\), \(\gamma\) = 90°).

Like cdAE1, cd\(\Delta 54AE1\) is a dimer consisting of an N-terminal interaction domain (residues 55–313) and C-terminal dimerization arms (residues 314–347) involved in domain swapping. The results indicate that the acidic N-terminal extension has little effect on the core structure of cdAE1, consistent with the first 54 residues operating as its own independent intrinsically disordered region involved in protein interactions with cytosolic proteins such as hemoglobin (56). There are subtle differences between the two monomers in the dimer that may be due to crystal packing. In chains A and P, the first residues visible in the electron density were Val57 and Lys56, respectively (Fig. 5A). In chain A, residues could be fit within the electron density to Ser350, but in chain P, the residues could be fit only to Gln348. In chain A, there is a disordered region from Ser181 to Asp183, resulting in loss of part of a hairpin loop that binds ankyrin (57). One other difference is in a loop region (residues 202–211) seen in the pH 4.8 structure that is more disordered in the pH 6.5 structure, extending further from residue Glu202 to Asp218, resulting in loss of a short helix (218; residues 212–220) visible at pH 4.8. The new crystal structure indicates that regions of cdAE1 involved in protein interactions are dynamic and can assume different conformational states at different pH values, perhaps folding up upon protein binding. Overall, the structure is very similar over...
residues 55–356 to the structure (Protein Data Bank code 1HYN) first published by Low and co-workers (24) (root mean square deviation was 1.14 Å over 530 aligned residues using SSM in Coot), showing that there is good agreement between the core protein structures obtained at pH 4.8 and 6.5 under two different crystallization conditions, crystal forms, and space groups (Fig. 5B). The differences in the structure of the loop regions involved in protein interactions are of significance because some regions folded and buried at low pH become unfolded and accessible at physiological pH.

Using the pH 6.5 crystal structure, the possible tunnel region around Arg283 is illustrated in Fig. 5, C and D. Arg283 is located in the middle of chain A (residues 278–290). As indicated by Chang et al. (23), Arg283 is in an occluded, solvent-inaccessible region of the protein. There is a hydrogen bond between the side-chain nitrogen of Arg283 to the side-chain oxygen of Glu85 (2.74 Å), which is located near the beginning of helix 7 (residues 84–88). In turn, the carbonyl side chain of Glu85 makes a hydrogen bond to the NH group of Arg283. Arg283 also makes two hydrogen bonds to the main-chain carbonyl of His101. Arg283 also makes a hydrogen bond with the hydroxyl group on Glu280 located three residues proximal on the same side of helix 7. Glu280 in turn makes hydrogen bonds to Asn87 and Ser100. Glu85 makes a hydrogen bond to Pro213 near the beginning of helical segment α6 (residues 212–220), which is destabilized at pH 6.5. A mutation of Arg283 to Ser would result in disruption of this elaborate H-bonding network and could destabilize the protein. Indeed, all attempts to crystallize the R283S or R283A mutants under conditions similar to the wild-type protein failed, indicating a disturbed protein structure. Therefore, we carried out a series of biophysical studies to determine the effect of the mutations on the stability of the isolated cytosolic domain.

**Sensitivity of cdΔ54AE1 Tunnel Mutants to pH and Urea Denaturation**—The cdAE1 undergoes dramatic changes with pH, transitioning from a compact structure under acidic conditions to a more extended and open structure at neutral and alkaline conditions (24, 26, 55, 58). Titration of cdΔ54AE1 showed a typical increase in intrinsic fluorescence going from acidic to alkaline pH arising from dequenching of clustered tryptophan residues (Fig. 6A). The R283S mutant underwent a similar transition but had a higher intrinsic fluorescence at low pH than the wild-type protein. This feature is consistent with a more open structure at low pH as was also observed for kidney cdAE1 (35). A similar effect was seen for the other mutants with a higher intrinsic fluorescence at low pH than wild type and an increase in fluorescence at alkaline pH (supplemental Fig. 1). The wild-type cdΔ54AE1 protein and the R283S mutant had identical CD spectra (Fig. 6B), indicating that this point mutation induced no major change in the secondary structure of the domain.

The sensitivity of wild type and tunnel mutants of cdΔ54AE1 to urea denaturation was examined at different pH values (supplemental Fig. 2). At pH 10.5 when cdΔ54AE1 has an extended conformation, urea resulted in protein unfolding with a decrease in intrinsic fluorescence at urea concentrations above 3 M. The intrinsic fluorescence of the tunnel mutants had a similar pattern, although a decline in intrinsic fluorescence was observed for all the mutants by 3 M urea, indicating a slightly lower stability. At pH 8.5, cdΔ54AE1 exhibited an initial increase in intrinsic fluorescence, peaking between 2 and 3 M.
urea and then declining at higher urea concentrations. The increase is due to dequenching of the tryptophan as the domain begins to unfold, whereas the decrease is due to exposure of the tryptophans to solvent as unfolding proceeds. The mutants showed a similar pattern with an increase in intrinsic fluorescence up to 2 M urea followed by a decrease. At pH 6.5, the Absence of a Substrate Access Tunnel in AE1

FIGURE 6. Effect of R283S mutation on the biophysical properties of cdΔ54AE1. A, effect of pH on the intrinsic fluorescence of cdΔ54AE1 and the R283S mutant. Both proteins showed an increase in fluorescence at alkaline pH with the mutant exhibiting a higher fluorescence at low pH values, consistent with a more open structure. B, circular dichroism (CD) spectra of cdΔ54AE1 and the R283S mutant in 100 mM NaCl, 20 mM Tris-HCl, pH 8.0. C, gel filtration profiles of cdΔ54AE1 and the R283S mutant. Samples were applied to an S200 HiLoad 16/60 column equilibrated in 100 mM NaCl, 20 mM Tris-HCl, pH 8.0. The major peak corresponds to the dimeric form of the proteins. AU, absorbance units.
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increase in intrinsic fluorescence for wild type and the tunnel mutants occurred at higher urea concentrations (5 M), consistent with a more stable structure at low pH. An intermediate effect was seen at pH 7.5. These denaturation studies support the view that the tunnel mutations affected the stability of the cytosolic domain.

The cdΔ54AE1 (monomer calculated molecular weight, 34,739) eluted as a major peak on an S200 16/600 gel filtration column with an apparent molecular mass of 122 kDa with a smaller amount of a higher molecular weight peak (Fig. 6C). The major peak corresponds to a dimer; the larger apparent molecular weight is due to the asymmetric nature of the dimer (55). The R283S mutant (monomer calculated molecular weight, 34,669) eluted as a major peak with a slightly larger apparent molecular mass of 143 kDa, consistent with a more asymmetric structure as well as an earlier peak ascribed to a tetramer. Light scattering measurements (supplemental Fig. 3) using a Malvern Viscotec low angle light scattering instrument connected to an S200 10/300 column showed that the wild-type cdΔ54AE1 dimer had a molecular mass of 73.5 kDa and a radius of hydration of 3.6 nm. The R283S dimer had a molecular mass of 71.4 kDa with a larger radius of hydration of 4.2 nm, indicating a more asymmetric structure. The mutant dimer tended to form tetramers upon rechromatography, forming a new peak with a molecular mass of 159 kDa and a radius of hydration of 6.7 nm with small amounts of higher oligomers eluting at the void volume, whereas the wild-type protein retained a stable dimer oligomeric structure. These results show that the R283S mutation had a destabilizing effect on cdΔ54AE1, resulting in a more asymmetric protein that is prone to aggregation, the likely cause of our inability to crystallize the mutants.

DISCUSSION

The report (23) of a bicarbonate tunnel within the cytosolic domain of NBCe1, a member of the SLC4 family of anion transporters, is intriguing. Therefore, we set out to determine whether a similar substrate access tunnel exists in human AE1. The cdAE1 is known to undergo major conformational changes with pH (26, 27) that could conceivably open or close the tunnel, providing a means to regulate anion transport in response to local changes in pH (59). In addition, the cdAE1 binds cytoskeletal proteins like ankyrin and protein 4.2, glycolytic enzymes, and hemoglobin (58). Interaction with these proteins could induce conformational changes in cdAE1 that in turn could affect anion transport. The fact that removal of the cytosolic domain does not affect transport (25) does not preclude the domain playing a role in regulating transport in the intact protein. Finally, the cytosolic domain of kAE1 is missing the first 65 residues found in AE1, including a central β-strand, resulting in a folded structure different from cdAE1 (35, 60). This suggests that kAE1 may bind a different spectrum of proteins and that a different regulatory mechanism could be at play in the kidney.

To test the hypothesis that the cdAE1 contains a substrate access tunnel, we constructed a full panel of mutations at residues by creating a less compact structure. This conclusion is supported by the gel filtration results that showed that the R283S mutant had a higher radius of hydration, consistent with a more asymmetric structure. The mutant was also prone to tetramer formation and aggregation, consistent with a less stable structure.

Four tryptophan residues in cdAE1 are responsible for the intrinsic fluorescence (Fig. 5C): Trp75 and Trp81 are buried, Trp94 is surface-exposed, and Trp105 is also exposed and hydrogen bonds to Asp346 in the opposite subunit. Asp346 in turn forms a water-mediated H-bond to His275, which forms a π stacking interaction with Trp105. Disruption of the interaction

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between Trp105 and Asp316 due to deprotonation of Asp316 allows cdAE1 to assume a more open dimeric structure at alkaline pH. Indeed, mutation of Trp105 or Asp316 revealed that a hydrogen bond between these two residues present at low pH is broken as the pH is raised, allowing the dimer to become more asymmetric (27). The collapse of the hydrogen-bonded network involving Arg283 leads to a less stable and less compact structure, affecting the packing of tryptophan residues and their exposure to solvent.

AE1 in the native membrane can be efficiently cross-linked by CuSO4/α-phenanthroline oxidation under alkaline conditions to a dimer by formation of a disulfide bond between Cys201 in one subunit and Cys317 in the other subunit (61–64). Upon proteolytic release from the membrane, the cysteine residues of cdAE1 form an intramolecular rather than an intermolecular disulfide bond under oxidizing conditions (62). Curiously, Cys201 and Cys317 are not close enough in the crystal structure at pH 4.8 or 6.5 to cross-link to each other within the monomer or between monomers. Cys201 is at the N-terminal region of a disordered loop (residues 202–220), whereas Cys317 is located in a turn between helix α9 and β11 in the dimerization arm. Thus, the crystal structures at pH 4.8 and 6.5 may not be adequate to reveal the dynamic states that cdAE1 attains in its native state that may be revealed by other techniques such as NMR or molecular dynamic simulations.

In this study, we showed that tunnel AE1 mutants could be functionally expressed in HEK-293 cells to a level similar to wild-type AE1. The crystal structure of cdΔ54AE1 obtained at pH 6.5 proved similar to the previous pH 4.8 structure except that some interacting loops in one subunit were more accessible and disordered at neutral pH. The native structure at pH 6.5 showed that Arg283 is in a solvent-occluded pocket and is intimately involved in an extensive hydrogen-bonded network with Glu85 and a number of neighboring residues (23). Examination of the crystal structure showed that these residues do not line an aqueous tunnel but rather form a “hydrogen-bonded network of polar residues” as originally described by Chang et al. (23). Mutation of Arg283 to Ser would be expected to have a disruptive effect on this network. Indeed, we observed that the R283S mutation had a destabilizing effect on the isolated cdΔ54AE1, resulting in a more open structure prone to aggregation. We have not been able to crystallize any of the tunnel mutants under conditions similar to cdΔ54AE1, confirming that the mutations affected the native structure of this domain.

The cytosolic and membrane domains of AE1 appear structurally and functionally independent. Trypsin cleavage of ghost membranes produces the cdAE1 as a soluble domain that retains its ability to interact with its protein partners (58) and the mdAE1 that is fully functional (25). The biophysical studies reported in this study showed that the mutations in the cytosolic domain destabilized the isolated domain and promoted its aggregation but did not impair the ability of the intact protein to traffic to the cell surface and transport anions. The mdAE1 was also able to traffic to the cell surface and was fully functional in transfected HEK-293 cells. Hereditary spherocytosis mutations in cdAE1 are impaired in Protein 4.2 binding but do not affect the ability of AE1 to traffic to the cell surface (17, 18). The R283S mutation did affect the stability of cdΔ54AE1 as predicted from its involvement in a hydrogen-bonded network in the crystal structure. Thus, in the case of AE1, mutations that destabilize the cytosolic domain do not affect the ability of the intact protein to traffic to the cell surface and assume its transport function. Although the cytosolic and membrane domains of AE1 operate quite independently, histidine residues within the larger cytosolic domain of AE2 act as a pH sensor that stimulates transport at alkaline pH, suggesting a regulatory role for the cytosolic domain (65–70).

We conclude that the cytosolic and membrane domains of human AE1 operate independently. The cytosolic domain of AE1 does not contain a substrate access tunnel necessary for transport activity, and therefore, such a tunnel is not an essential feature of the SLC4 family of bicarbonate transporters.

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