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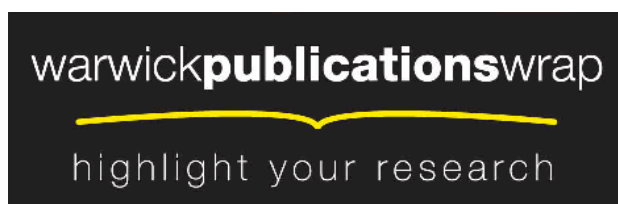
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## **Crystal structure of the anion exchanger domain of human erythrocyte Band 3**

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One sentence summary: Structure of erythrocyte Band 3 reveals mutation locations leading to red cell diseases.

## **Abstract**

Anion Exchanger 1 (AE1, SLC4A1), also known as Band 3 plays a key role in the removal of carbon dioxide from tissues by facilitating the exchange of chloride and bicarbonate across the plasma membrane of erythrocytes. An isoform of AE1 is also present in the kidney. Specific mutations in human AE1 cause several types of hereditary hemolytic anemias and/or distal renal tubular acidosis. Here, we report the crystal structure of the Band 3 anion exchanger domain (AE1<sub>CTD</sub>) at 3.5 Å. The structure is locked in an outward-facing open conformation by an inhibitor, H<sub>2</sub>DIDS. Comparing this structure to a substrate bound structure of the UraA uracil transporter in an inward-facing conformation allowed us to identify the anion-binding position in the AE1<sub>CTD</sub> and to propose a possible transport mechanism revealing why selected mutations lead to disease.

Efficient delivery of oxygen to tissues and removal of carbon dioxide in blood is fundamental for respiration. The red blood cells or erythrocytes provide the principal route to achieve this. Anion exchanger 1 (AE1), also known as Band 3 or SLC4A1 predominates in the erythrocyte ghost membrane and constitutes 30% of its protein (1). It plays a major role in gas transport by converting the CO<sub>2</sub> generated to the more soluble bicarbonate form (HCO<sub>3</sub><sup>-</sup>), increasing the CO<sub>2</sub> bearing capacity of the blood. Carbon dioxide rising from metabolic processes in the tissues diffuses into the red blood cells. Here it reacts with water in a reversible process catalysed by carbonic anhydrase II to form HCO<sub>3</sub><sup>-</sup> and protons (2). As the bicarbonate concentration in the erythrocyte increases, the anions are transported by AE1 out into the blood plasma in an electroneutral exchange for chloride ions. With approximately 10<sup>6</sup> AE1 molecules per cell (1) and each single protein transporting 4-5 x 10<sup>4</sup> ions per second (3,4), transport is extremely fast and approximately 90% of CO<sub>2</sub> is taken from the tissues to the lungs as bicarbonate. Within the erythrocyte the net result is the accumulation of protons from the hydration of CO<sub>2</sub>. Due to the Bohr effect, the low pH causes hemoglobin to release oxygen, which can then diffuse into the tissues. When the blood reaches the lungs, the process is reversed and CO<sub>2</sub> is exhaled.

Erythrocyte AE1 is the most abundant and widely studied anion exchanger but AE1 and its close homologues AE2 and AE3 are found in diverse tissues (5,6), playing important roles in the regulation of intracellular pH, cell volume and membrane potential through HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> exchange. AE1 is highly expressed as an N-terminally truncated form in kidney, where it is instrumental in the reabsorption of bicarbonate (7). Many morphological and anemic disorders to the erythrocytes and distal renal tubular acidosis in kidney are caused by inherited mutations in AE1 (8).

Human erythrocyte AE1 is a 110kDa glycoprotein. It is built from two domains (9), a cytosolic N-terminal domain (residues 1-360) and an integral membrane domain (residues 361-911). Anion exchange is catalyzed by the C-terminal domain (10-12). However, only the crystal structure of the cytosolic N-terminal domain (13), important as an anchoring point for other proteins including the scaffolding protein ankyrin (14, 15) and deoxy-hemoglobin, has been determined (16,17). A wealth of biochemical experiments, including cysteine scanning mutagenesis (18-23) and N-glycan insertion mutagenesis (24,25), have been used to derive topology models of AE1. The only 3-dimensional structural information available to date for the transmembrane domain is from electron microscopy (26,27) with the best maps at a resolution of 7.5Å calculated from 2-dimensional crystals (28). As such, the membrane topology, substrate recognition and the anion-transport mechanism of this fundamental protein remain unclear and diverse models have been proposed (28-31). Here we report the crystal structure at 3.5Å resolution of the membrane domain of human AE1 locked in an outward-facing open conformation by a covalently bound H<sub>2</sub>DIDS-inhibitor, in complex with a Fab fragment from a monoclonal antibody.

The C-terminal, anion exchanger domain of AE1 (AE1<sub>CTD</sub>), with the N-terminus cleaved by trypsin, was purified directly from white ghost membranes of human erythrocytes and was treated with H<sub>2</sub>DIDS (4,4-diisothiocyanatodihydro-stilbene-2,2-disulfonic acid), a disulfonic stilbene derivative that irreversibly inhibits anion exchangers by covalently binding to the protein and blocking the transport cycle (32,33). Two steps were required to obtain well diffracting crystals. Firstly the transporter was deglycosylated with N-glycosidase F. Secondly, the protein was cocrystallised with a monoclonal antibody that binds tightly to a conformational epitope of AE1<sub>CTD</sub>. This was selected from a panel of antibodies raised by the inoculation of mice with budded

baculovirus displaying AE1<sub>CTD</sub> (34). The structure was solved using MIRAS in combination with non-crystallographic symmetry averaging and refined at a resolution of 3.5Å to an R-factor of 27.4% and R-free of 29.0% (Tables S1 and S2) (32). The final model contains all residues from 381 to 887 apart from three loop regions (553-567, 640-649, 742-753). The asymmetric unit of the crystal contains two dimers of AE1<sub>CTD</sub> with one Fab fragment bound on the outer side of each protomer (Figs. S1 and S2).

Each protomer comprises 14 transmembrane (TM) segments (Fig. 1) and has dimensions of approximately 60 x 60 x 50 Å. The lengths of the individual TMs vary from 18 to 42 Å. Like many other secondary transporters, the structure is built from two repeats inverted in the plane of the membrane (Fig. S3). In AE1<sub>CTD</sub>, the repeat is made of 7 TMs with the third TM of each repeat only partially spanning the membrane before the helical structure unravels. Though it is difficult to superimpose all TMs from the inverted repeat as a unit, treating it as a two-component module TM1-4 can be superimposed on TM 8-11 (root-mean-square deviation (r.m.s.d.) of 2.1Å for 62 out of 103 C $\alpha$  atoms; see methods) and TM 5-7 onto TM 12-14 (r.m.s.d. of 2.1Å for 53 out of 100 C $\alpha$  atoms) (Fig. S3B).

The inverted repeat units intertwine to form two structural domains separated by a cleft on the extracellular side of the protein (Fig. 1A). We define these structural domains as the Core (TMs1-4 and 8-11) and the Gate (TMs 5-7 and 12-14), domains following the convention of UraA (35,36). Within the core domain, the N-termini of the two half-helices (TMs 3 and 10) face one another at a distance of ~10 Å giving the appearance of a continuous helix (Figs. 2A, B and S2B).

The overall fold of AE1 is very similar to the structure of the uracil transporter UraA (35) although they have only 12% sequence identity as aligned using the structures (Fig. S4). Structural similarity was previously suggested based on threading

combined with mutagenesis experiments (29). UraA is also made of two 7 TM repeat units forming two domains. As shown in Figure 2, the Core domains of AE1<sub>CTD</sub> and UraA are similar and can be superposed with an r.m.s.d. of 1.8 Å for 145 of 268 C $\alpha$  atoms (1.9 Å for 131 out of 190 C $\alpha$  atoms if only the TM segments are considered; Fig. S5). The Gate domains of the two proteins also have the same topology but it is more difficult to superpose these domains (r.m.s.d. of 2.0 Å for only 55 out of 166 C $\alpha$  atoms) (Fig. 2, C and D and Fig. S5). This is, in part, because the relative positions of the three pairs of helices (TMs 5 and 12, TMs 6 and 7 and TMs 13 and 14) are different between AE1 and UraA. As the gate domains are directly involved in substrate binding, as discussed below, the structural variation could reflect the different substrates of the two proteins.

AE1<sub>CTD</sub> is a physiological dimer (37) but dimerization is not necessary for transport (38,39). The dimer in the crystal consists of two AE1<sub>CTD</sub> monomers with 1092 Å<sup>2</sup> of surface area buried at the interface (32). Monomers are related by a 2-fold axis parallel to the membrane normal, consistent with dimer formation in the erythrocyte membrane (Fig. 1B). There is no obvious difference between the two dimers seen in the crystal at the current resolution. The interaction between subunits is formed exclusively through residues on the Gate domains including TMs 5, 6 and 7 (Fig. 1B).

The Fab fragments bind solely to the Core domain of each AE1<sub>CTD</sub> (Figs. S1 and S2). The interactions between AE1<sub>CTD</sub> and the Fab are identical for the four molecules of the asymmetric unit. The predominant interactions are between the heavy chain of the Fab and the C-terminal end of TM 3, the following loop and the loop before TM 8 (Fig. S1). The light chain of the Fab interacts with the end of TM 1 and the following loop.

H<sub>2</sub>DIDS covalently crosslinks Lys539 and Lys851 (40) and, in the electron density maps, there is non-protein density between them consistent with the inhibitor (Fig. S6). However, part of the H<sub>2</sub>DIDS density is ill-defined and it is possible that either the link



may have been damaged by radiation or the cross-linking may not have been carried out to completion (Fig. S6A,B). The H<sub>2</sub>DIDS molecule spans TMs 5 and 13 of the Gate domain (Fig. 2A) at the entrance of a large cavity 15 Å wide, 7 Å long and 11 Å deep on the extracellular side of the protein between the Core and Gate domains (Figs. 3A, S6, S7 and S8). The cavity is formed by TMs 1, 3, 5, 13 and 14 and has predominantly hydrophobic walls with polar residues near the entrance and at the bottom.

Mutagenesis studies of mouse AE1 demonstrate that the protein is still capable of Cl<sup>-</sup> self-exchange when the lysine residues interacting with H<sub>2</sub>DIDS are mutated (41) suggesting that the inhibitor does not bind in the same place as the substrate. Under the H<sub>2</sub>DIDS molecule the cavity opens out slightly (18 Å) where the N-termini of TMs 3 and 10 meet (Fig. 3). This region has been suggested as a cation selectivity filter (23). In UraA, uracil binds in the space between the positive dipoles TMs 3 and 10. The uracil interacts with two glutamate residues, one on TM8 (Glu241) and the other on TM 10 (Glu290) (Fig. 3B) (35). In AE1<sub>CTD</sub>, Glu681 and the positively charged Arg730 take the positions of these two glutamates on TMs 8 and 10, respectively (Fig. 3B). Both of these residues are conserved in AE1, and anion exchange is lost if either is mutated (19, 42-44). Though there is no apparent density for a substrate and substrate binding could be blocked by bound H<sub>2</sub>DIDS, a bicarbonate ion can be placed between the positive dipoles of TMs 3 and 10. The negatively charged bicarbonate could interact with the positively charged Arg730, and could hydrogen bond to Glu681. The anions may also interact directly with the amide protons at the N-termini of TMs 3 and 10 (Fig. 3B). Consistent with anion binding in this space, mutagenesis studies indicate that only a conservative mutation to a threonine (30) or cysteine (29) is tolerated at Ser465 at the N-terminus of TM3. Mutation to the larger isoleucine (29), asparagine or aspartic acid (30) abolishes transport. The arrangement of an anion between the positively charged dipoles of half-

helices (TMs 3 and 10 in AE1) with a negatively charged residue nearby (Glu681 in AE1) is similar to the selectivity filter of the CLC chloride transporter although the topology of the two proteins is completely different (Fig. S9) (45).

AE1 exhibits modes of transport other than the physiological 1:1 exchange of bicarbonate and chloride ions. It can also conduct anions (46) or cotransport protons to drive the uptake of divalent sulfate (47) or chloride ions (48). These ions can easily be accommodated in the basic cavity (Figs. S7 and S8). Glu681 on the translocation pathway is a potential proton acceptor during H<sup>+</sup>/SO<sub>4</sub><sup>2-</sup> cotransport as modification to an alcohol (Glu681OH) or mutation to a glutamine leads to a highly proton independent SO<sub>4</sub><sup>2-</sup>/Cl<sup>-</sup> exchange, and electroneutral SO<sub>4</sub><sup>2-</sup>/SO<sub>4</sub><sup>2-</sup> exchange (42, 49-50).

Specific mutations in the AE1<sub>CTD</sub> domain (Fig. S10), are related to red cell diseases such as spherocytosis (51), stomatocytosis (52) and southeast asian ovalocytosis (SAO)(53). Some of these mutations, particularly those leading to spherocytosis, cause misfolding of the protein whereas others exhibit abnormal transport kinetics. Some of the mutations in the transport domain are listed in Table S3 and shown in Figs. 4 and S11, S12. An increase in membrane permeability to monovalent cations caused by mutations in AE1 has been seen in a number of human pedigrees with dominantly inherited hemolytic anemia (22, 44, 52). These mutations mostly occur on the cytoplasmic half of the Core domain (Fig. 4). They include mutation of Arg730, the putative bicarbonate-binding residue, to Cys as well as two other mutations on the half-helix TM10 (Ser731 to Pro, His734 to Arg). The deletion of residues 400 to 408 leading to SAO also causes a cation leak in intact red cells (54). These residues reside at the N-terminus of TM 1 where it interacts with TM 7 of the Gate domain (Figs. 4 and S12) and would presumably alter the structure of the Core domain as well as the interaction between AE1<sub>CTD</sub> and the N-terminal cytoplasmic domain. It is

interesting that this deletion may confer protection against cerebral malaria (54). Fewer mutations have been reported in the Gate domain.

The structure of AE1<sub>CTD</sub> reported here is in an outward-facing conformation (Fig. 1 and 3). AE1<sub>CTD</sub> is reported to undergo large conformational changes during transport in line with the alternating access mechanism (55, 56). By comparing the structures of outward-facing AE1<sub>CTD</sub> and inward-facing UraA with a bound substrate (35), we can gain some insight into the mechanism (Figs. 3A and S13).

The Core domain of these two proteins, including the uracil binding site in UraA, is very similar (Figs. 3B and S5). The major difference between the two structures is seen in the relative positions of the Gate and Core domains (Fig. 2, C and D and Fig. S5). The rotation of one domain against another as the transporter moves from outward to inward facing, as seen in Fig. S5, is very similar to the predominant movements seen for other families of transporters including the LeuT family transporters to which there is some structural similarity (Fig. S14) (36, 57-61). Fig. S13 shows a possible transport mechanism of AE1. Starting from an outward-facing open conformation of the protein, chloride at a high concentration in plasma, binds to the anion-binding site. This causes some local conformational changes of the Core domain enabling this domain to rotate against the Gate domain to form the inward-facing structure. At this point chloride diffuses out and is replaced by bicarbonate to reverse the process. This is consistent with various results in kinetic studies indicating that chloride and bicarbonate ions share the same binding site (4). The structure of the human AE1<sub>CTD</sub> and the proposed transport mechanism provides a scaffold through which to understand the many mutations in the protein that lead to diseases.

## Supplementary Materials

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Materials and Methods  
Figs. S1 to S15  
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Tables S1 to S3

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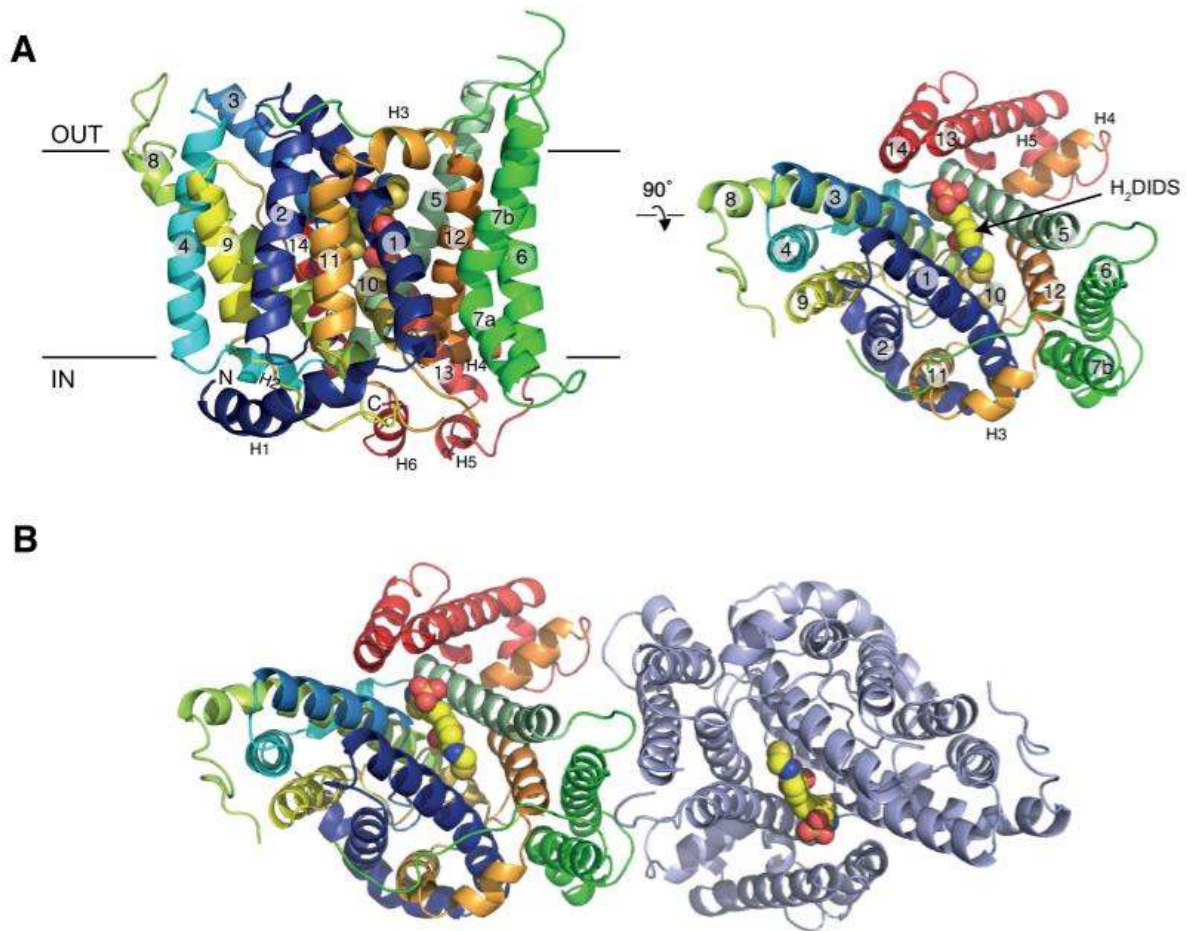
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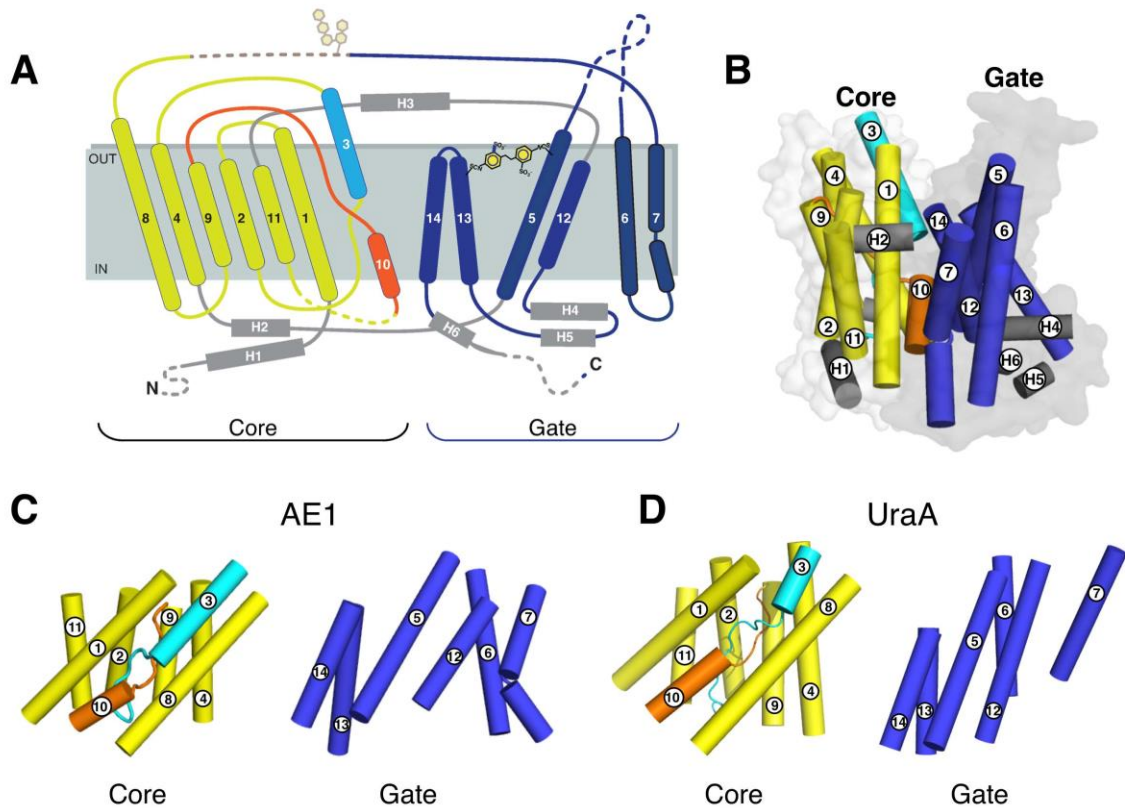
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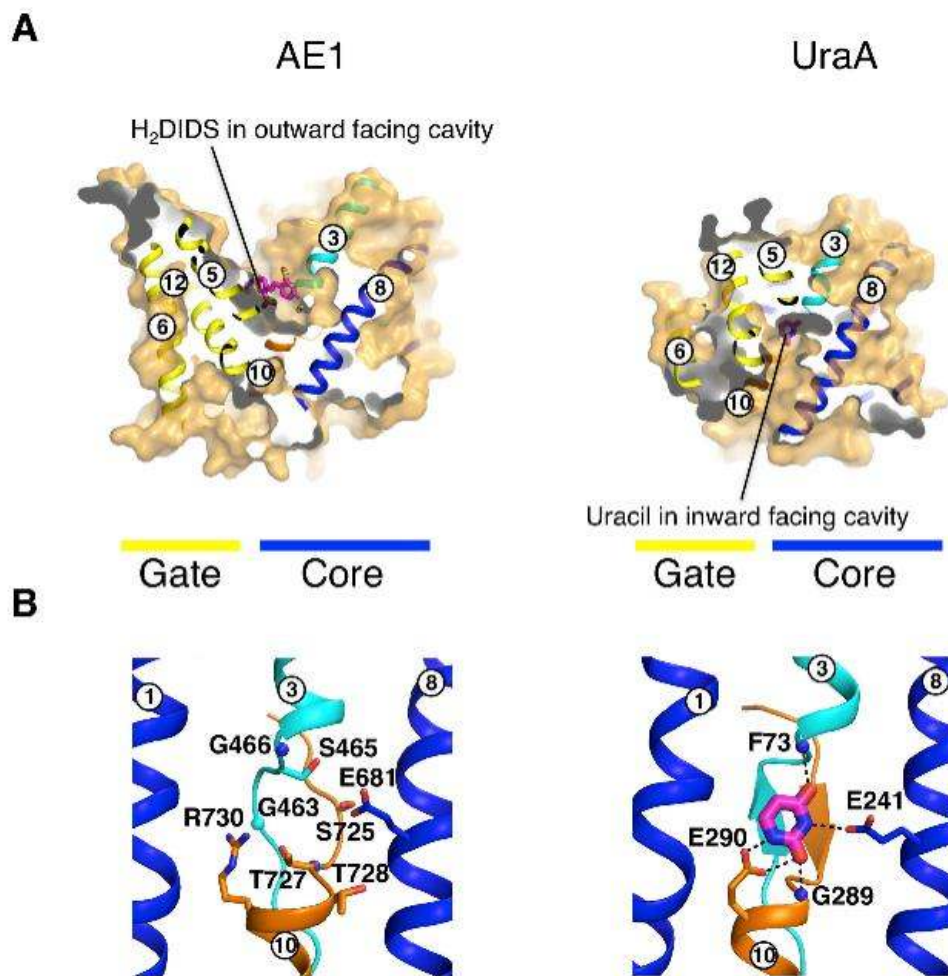
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**Fig. 1. Structure of AE1<sub>CTD</sub> in an outward-facing conformation. (A)** View of the structure in the plane of the membrane (left) and from the extracellular side of the membrane (right). The cartoon representation of the structure has been colored from blue at the N-terminus to red at the C-terminus. The H<sub>2</sub>DIDS is depicted by spheres (C: yellow, S: dark yellow, O: red and N: blue). Six short helices on the membrane surface are shown as H1 – H6. **(B)** Structure of the dimer viewed from the extracellular side. The color coding of the left monomer is the same as in (A). The right monomer is shown in grey.



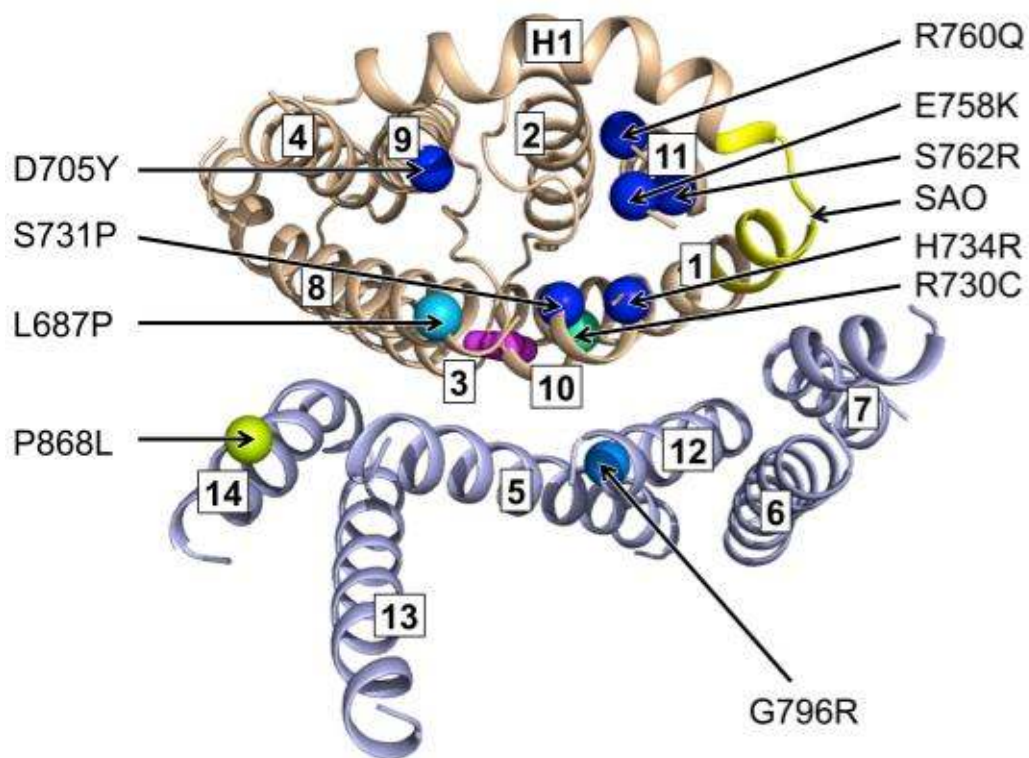
**Fig. 2. Structural framework of AE1<sub>CTD</sub> and comparison with UraA.** (A) Topology and (B) overall structure of AE1<sub>CTD</sub> viewed in the plane of the membrane. TM3 is colored in cyan and TM10 in orange. The other transmembrane helices of the Core domain are colored yellow and those of the Gate domain blue. (C) The Core and Gate domains of AE1. Left: the Core domain viewed from the Gate domain. Right: the Gate domain viewed from the Core domain. Coloring as in (A). (D) As (C) but for UraA, (PDB accession code 3QE7). The two proteins were aligned on their respective Core domains.



**Fig. 3.**

**Outward- and inward-facing cavities and substrate binding sites in AE1 and UraA.**

**(A)** Surface representations of the outward-facing structures of the substrate-free AE1<sub>CTD</sub> complex with H<sub>2</sub>DIDS (left) and the inward-facing structure of UraA (PDB accession code 3QE7) with uracil (right). These are the views in the plane of the membrane. The surfaces are semi-transparent and slabbed to show the positions of the transmembrane helices, which are colored as in Fig. 2. The H<sub>2</sub>DIDS and uracil are shown in magenta on the left and right panels, respectively. **(B)** Comparison of the putative anion binding site in AE1 (left) with the Uracil binding site in UraA (right). The coloring is as in (A). Glu241 and Glu290 of Uracil correspond to Glu681 and Arg730 of AE1 respectively. The dark blue spheres represent the amide nitrogens of the depicted residues and the light blue sphere the C $\alpha$  atom of Gly463.



**Fig. 4. Some mutations reported to cause diseases, plotted on the structure of AE1<sub>CTD</sub>.** The Core domain is colored coral and the Gate domain light blue. The view is from the cytoplasmic side of the membrane. The deletion mutation of residues 400-408, which causes SAO is shown in yellow. Point mutations are shown by spheres with coloring according to conservation among the 10 human SLC4 family transporters. This was generated with the ConSurf server (<http://consurf.tau.ac.il/>) (62) using AE1-4, NBCe1-2, NBCn1, NDCBE, NCBE and BTR1 and colored according to the colours of the rainbow with blue, highly conserved and red, poorly conserved. R730 is only conserved amongst the electrogenic family members. The details of mutations are described in Table S3 and Figs. S11 and S12. The position of uracil in the UraA structure, corresponding to the possible anion binding site in AE1, is shown with magenta spheres.