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Band 3 Function and Dysfunction in a Structural Context

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Band 3 Function and Dysfunction in a Structural Context

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Purpose of review: Current research on the human Band 3 glycoprotein, the red cell chloride/bicarbonate anion exchanger (AE1), is highlighted and placed within a structural context.

Recent Findings: The determination of the crystal structure of the membrane domain of human Band 3, the founding member of the SLC4 family of bicarbonate transporters, is a major breakthrough towards understanding the mechanism of action of this membrane transport protein, its interaction with partner proteins, and how mutations linked to disease affect its ability to fold and function.

Summary: Band 3 contains 14 transmembrane (TM) segments arranged in a 7+7 TM inverted repeat topology common to all members of the SLC4 family and the unrelated SLC26 anion transporter family. A functional feature of this fold is the presence of a core and a gate domain: the core domain contains two short TM helices (TM3 and 10) that face each other in the middle of the membrane with the positive N-terminal helix dipoles creating the anion binding site, while the gate domain forms the dimer interface. During transport, the movement of these two domains relative to each other provides the intracellular and extracellular compartments with alternating access to the central anion binding site.

Keywords: anion transport, Band 3, membrane proteins, SLC4, trafficking

Introduction

Band 3, also known as Anion Exchanger 1 (AE1) or Solute Carrier 4A1 (SLC4A1), is responsible for the electroneutral exchange of chloride and bicarbonate across the red cell membrane, a process necessary for efficient transport of CO_2 during respiration. Band 3 is also important for maintaining red cell shape, anchoring the actin-spectrin cytoskeleton at the membrane. A truncated kidney anion exchanger 1 (kAE1), lacking the first 65 residues, is expressed in the basolateral membrane of α-intercalated cells, where it plays a role in bicarbonate reabsorption into the blood to facilitate acid secretion into the urine. Genetic defects in the *SLC4A1* gene lead to red blood cell diseases, which include hereditary spherocytosis (HS), hereditary stomatocytosis (HSt), and Southeast Asian ovalocytosis (SAO), as well as the kidney disease distal renal tubular acidosis (dRTA).

Human Band 3 is an abundant 911-residue glycoprotein comprised of two domains: an N-terminal cytosolic domain (cdAE1) that anchors the cytoskeleton at the membrane and interacts with numerous erythrocyte proteins including deoxyhemoglobin [1] and a C-terminal membrane domain (mdAE1) that performs anion exchange [2]. Through a cytosolic C-terminal tail, the membrane domain interacts with carbonic anhydrase II (CAII), which interconverts carbon dioxide and bicarbonate, to form a bicarbonate transport metabolon [3,4]. Band 3 exists as a mixture of dimers and tetramers in membranes and detergent solutions [5]. These oligomers form interaction hubs around which integral and peripheral membrane proteins of the red blood cell are organized [6].

A variety of methods have been used to elucidate the transmembrane topology of Band 3, including proteolysis, chemical labeling, epitope mapping, scanning N-glycosylation and cysteine mutagenesis, and fragment-complementation assays [7**]. While these experiments indicated that mdAE1 crosses the membrane up to 14 times, the topology around putative spans 9 to 12 remained unclear. The crystal

structure of cdAE1 was determined in 2000 [1], but mdAE1 resisted high-resolution structural analysis for a further 15 years [8**]. Crystallization of dimeric human mdAE1 (Fig. 1) was enabled by trypsin cleavage and deglycosylation, use of a monoclonal Fab that bound a conformational epitope, and locking of the structure in an outward-facing open conformation with H_2DIDS, a competitive inhibitor of anion transport.

The membrane domain of Band 3 has a 7+7 TM inverted repeat structure

The crystal structure of mdAE1 confirmed that Band 3 contains 14 α-helical transmembrane (TM) segments that consist of two inverted seven TM-repeats (TM1 to 7 and TM8 to 14) (Fig. 2a). The TM segments have a complex topology and are intertwined to form two sub-domains: the core and gate domains (Fig. 2b). TM1 to 4 and 8 to 11 make up the core domain, and TM5 to 7 and 12 to 14 the gate domain (Fig. 2c). This folding pattern, known as the 7+7 TM inverted repeat fold [9*], differs from the major facilitator superfamily (MFS) of transporters that have a much simpler 12 TM topology arranged in two linear domains (TM1-6 and TM7-12) connected by a cytosolic linker [10,11*]. The 7+7 TM inverted repeat topology appears to be common to the SLC4 family proteins, as suggested by recent structures of proteins related to human SLC4A11 [12*,13*]. This fold was first observed in the bacterial proton-uracil symporter UraA, a member of the SLC23 nucleobase transport family [14*], and subsequently in the fungal proton-purine symporter UapA [15*] and a bacterial SLC26 anion transporter [16*].

Helix dipoles comprise the anion binding site

A unique feature of 7+7 TM inverted repeat proteins is the presence of two pseudo-symmetry related half-helices, TM3 and TM10, that face each other in the middle of the lipid bilayer [9*]. In UraA and UapA, substrates are bound between the N termini of these two half-helices [14*,15*]. In mdAE1, the

anion-binding site is also found between the N-terminal ends of TM3 and 10 located in the core domain (Fig. 3a). The anion is held in place by the positive helical dipoles [17]. Transport also involves Arg730 from TM10 and Glu681 from TM8 (Fig. 3a). Arg730 forms part of the anion-binding site and interacts with one of the sulfates of H_2DIDS, while Glu681 points towards the anion-binding site and may act as an anionic gate. Consistent with this mode of substrate binding, mutation of Arg748 in mouse Band 3 (equivalent to Arg730) impairs anion transport [18]. Of note, Glu681 has been identified as the proton-binding site for proton-sulfate/chloride exchange by Band 3 [19]. In the sodium-bicarbonate co-transporting members of the SLC4 family, Glu681 is replaced by Asp, suggesting that this Asp likely provides the sodium-binding site in these proteins [20].

Disease-causing Band 3 mutations

The crystal structure of mdAE1 revealed the location of a variety of mutations that cause the diseases HS, HSt, and dRTA [7**]. Many of these mutations cause misfolding and/or aberrant trafficking of the protein, affecting Band 3 localisation during red cell development or targeting to the basolateral surface of kidney epithelial cells. While most mutations cause standalone trafficking defects that impact only one allele and lead to recessive disease, some mutations can lead to mis-trafficking of the wild type protein by hetero-dimerization causing a dominant disease phenotype [7**]. Band 3 trafficking is also influenced by additional factors, including Glycophorin A (GPA) and ER chaperones. GPA facilitates trafficking of Band 3 to the cell surface and can rescue certain Band 3 mutants, including Band 3 SAO [21]. ER chaperones involved in the folding of glycoproteins, like calnexin, are selectively removed during terminal erythropoiesis while inhibition of these chaperones in kidney cells can rescue trafficking of certain kAE1 mutants [22,23]. Specific mutations in Band 3 can cause HSt by inducing cation passage in the protein; many of these mutations are clustered at the interface between the core and gate domains near the anion binding site (Fig. 3b) [7**].

Ion transport mechanism of mdAE1

The core and gate domains are separated by a V-shaped cleft that opens to the extracellular side of the membrane (Fig. 4a). The substrate-binding site lies at the vertex of the cleft (Fig. 4a), and substrate passage to this region is blocked when the inhibitor H_2DIDS is bound (Fig. 2c). Substrate binding residues are exclusively in the core domain (Fig. 3a), while the DIDS-reactive Lys539 and Lys851 are in the gate domain on TM5 and 13, respectively (Fig. 2c).

Anion exchange is achieved through a relative movement of the core and gate domains, providing the substrates with alternating access to either side of the membrane [24]. While the precise mechanism of anion transport remains unclear, there are three prevailing models for alternating access (Fig. 4b) [25]. In the "rocking switch model" both core and gate domains move relative to the lipid bilayer to provide the alternating access. In the "rocking bundle model" alternating access is accomplished through the movement of one of the domains against the other, which remains immobile. Finally, in the "elevator transport model" one domain moves against the other to change the depth of the substrate-binding site in the membrane. Structures of other 7+7 TM inverted repeat transporters showed that they adopt inward-facing or intermediate occluded conformations, in contrast to the outward-facing mdAE1 [12*,13*,14*,15*,16*,26**]. Thus far, the structure of only one member of the 7+7 TM inverted repeat family, UraA, has been reported in two states: inward-facing and occluded [26**]. Comparison of these two conformations suggests a hybrid rocking-bundle elevator mode of transport, in which alternating access is achieved through a combination of a relative movement of the core against the gate, local rearrangement within the gate, and concomitant translocation of the substrate. However, a model of inward-facing mdAE1 built using a technique known as repeat-swap homology modelling, in which the conformation of the two pseudo-symmetry related repeats are exchanged [27*,28], suggests that mdAE1

uses only an elevator-like mechanism for anion transport. Further experiments are needed to discern between the possible modes of transport by mdAE1 and related family members.

The crystal structure provides context for other functional elements of Band 3

The N- and C-terminal regions of mdAE1 are in the cytoplasm and contain two amphipathic α-helices, H1 and H6, that lie along the inner membrane surface (Fig. 4c). H1 (residues 383-401) precedes TM1, connecting the transmembrane region to the cytosolic domain of Band 3. A nine-amino acid deletion (Ala401-Ala409) in this region causes SAO [29], potentially altering the relative orientation of the cytoplasmic and membrane domains. H6 follows TM14 and links it to the C-terminal CAII-interacting region of Band 3.

The membrane domain of AE1 contains four additional amphipathic α-helices (H2-H5) that connect TM segments (Fig. 4c). H2 lies on the cytoplasmic face of mdAE1 between TM4 and 5 and H3 lies on the extracellular side between TM11 and 12. Both H2 and H3 connect the core to the gate domain, and the repeat-swap homology model of inward-facing mdAE1 suggests that H2 and H3 undergo a rearrangement to facilitate mdAE1 conformational changes during anion transport [27*].

The mdAE1 and cdAE1 can be separated by mild protease treatment of red cell ghosts and are thought to function independently [2], unlike other SLC4 proteins in which the cytoplasmic domain is required for activity [30,31]. Analysis of purified bovine Band 3 by negative-stain electron microscopy suggested that the membrane domain and cytosolic domain are connect by a 3 nm flexible linker [32*]. In contrast, a model of full-length human Band 3 based on the crystal structures of mdAE1, cdAE1, and zero-length cross-linking analysis of red blood cell membranes suggested a more compact arrangement within the intact protein, as well as an interaction between cytoplasmic loops of the membrane domain and the cytosolic domain [33*]. This interaction is proposed to hold the cytosolic domain in place and out of the

143 way of the substrate exit pore on the cytoplasmic side of mdAE1 [33*], supporting the notion that Band
144 3 does not contain a substrate access tunnel in its cytosolic domain [34].

145 Interestingly, the presence of a discontinuous SH2 domain in Band 3 that regulates anion transport by a
146 phosphorylation-dependent mechanism was reported recently [35**]. A conserved phospho-tyrosine
147 binding motif was detected within H2 that interacts with phosphorylated cdAE1, disrupting Band 3-
148 cytoskeletal interactions and destabilizing the red blood cell membrane. This intramolecular
149 rearrangement would require that H2 partially unfolds to interact with phosphorylated cdAE1, and is
150 consistent with the notion that H2, which links the core and gate domains, is involved in conformational
151 changes associated with anion transport [27*]. Using Syk kinase inhibitors to prevent tyrosine
152 phosphorylation of Band 3 and increase erythrocyte membrane strength reduced *Plasmodium falciparum*
153 egress in parasitized red blood cells, thus providing a potential therapeutic avenue to inhibit malaria
154 progression [36].

155 **Band 3 dimer and tetramer structures**

156 The structure of mdAE1 showed that dimerization is mediated exclusively by the gate domain, through
157 interactions between TM5 and 6 from each subunit, and some residues from TM7, H4, and the loop
158 connecting H4 to H5 (Fig. 1). Interfacial lipids have been shown to be important for oligomerization of
159 many membrane proteins [37*] but their role in stabilization of the Band 3 dimer is unclear. While both
160 UraA and UapA require dimerization for transport activity [15*,26**], studies with inhibitors have
161 shown that each subunit of Band 3 can operate independently [38]. Indeed, co-expression of wild-type
162 Band 3 and a non-functional SAO Band 3 mutant results in the surface expression of a heterodimer that
163 retains almost 50% of wildtype activity [29]. Furthermore, it has been shown that the region around
164 TM6-7, which is involved in dimerization, was dispensable for Band 3 function [39,40].

Band 3 tetramers are dimers of dimers stabilized by binding of ankyrin to each tetramer [5,41]. Cross-linking analysis provides a model for Band 3 tetramer formation involving Glu272 and Lys353 from the cytosolic domains, and revealed a large interaction interface between Band 3 and ankyrin [33*]. Additionally, this analysis showed that GPA interacts with cdAE1 [33*], which also interacts with Glu658 in TM8/9 of mdAE1 to create the Wright blood group antigen [42]. Additional interactions were proposed for cytosolic loops of mdAE1 with protein 4.1 and protein 4.2, two adaptor proteins involved in maintaining cytoskeletal interactions. However, the significance of protein 4.2-mdAE1 associations are unclear as an enhancement effect on Band 3 anion exchange by protein 4.2 was only observed in the presence of the Band 3 N terminus that contains the main protein 4.2 binding site [43].

Band 3 is a protein interaction hub

The human Band 3 tetrameric macro/multiprotein complex, known as the Band 3 complex in early literature, consists of a tetramer of Band 3 bound to ankyrin, protein 4.2, and GPA. Ankyrin and protein 4.2 binding to Band 3 facilitates its association to the erythrocyte spectrin cytoskeleton. The Rhesus protein sub-complex comprising Rh, RhAG, LW, CD47, and Glycophorin B has also been shown to associate with the tetrameric complex [44]. In contrast, dimeric Band 3 is thought to be either freely mobile in the membrane or associated within an actin-junctional complex, the composition of which varies between species [44]. In humans, this junctional complex consists of Band 3 dimers, protein 4.1, adducin, dematin, GPC, p55, Rh, and also GLUT1 [44,45].

Several of the integral and peripheral membrane proteins that associate with Band 3 also influence its function and/or trafficking, such as GPA, which affects both [21]; carbonic anhydrase II, which forms a metabolon with Band 3 [3]; stomatin, which increased Band 3 transport activity [46]; and protein 4.2, which increased Band 3-specific chloride influx in Xenopus oocytes [43] but decreased activity in

reconstituted liposomes [47]. Of note, Aquaporin-1 was recently reported to interact with Band 3 [48], suggesting the existence of a super-complex involving Band 3, deoxyhemoglobin, CAII, and aquaporin-1 to channel substrates to and from the transporters and their cognate enzyme. How Band 3 associates with so many proteins, and which regions of Band 3 are involved, still needs to be fully delineated. However, with many copies of Band 3 (1.2×10^6) available in each cell it is likely that there are many flavours of Band 3 complexes.

Future prospects

The crystal structures of cdAE1 and mdAE1 have provided valuable molecular insights into Band 3 function and dysfunction, yet the molecular mechanism of substrate transport and Band 3's interaction with other proteins remains elusive. Structures of the inward-facing state of mdAE1 would reveal the extent of the conformational changes associated with transport. A high-resolution structure of intact Band 3 is needed to show the relative orientation of cytosolic and membrane domains and studies of the dynamics of this interaction are needed. Single-particle electron cryo-microscopy (cryo-EM), in particular, holds considerable promise as recent improvements in microscopes and image processing have led to high-resolution structures of complex membrane transport systems [49,50]. Molecular dynamics (MD) simulations of Band 3 in complex lipid bilayers will allow the conformational changes associated with transport to be modelled in a membrane environment [7**]. MD simulations have recently been used to determine the dynamics the Band 3 TM1 signal-anchor that is responsible for integrating the protein into the ER membrane [51]. Finally, the Band 3 structure has been used to build models of other members of the human SLC4 and SLC26 transporter families and to understand the effect of disease-causing mutations on protein folding and function [7**,52*,53, 54]. We can now look forward to the challenge of determining how this transporter works at the molecular level and how it

interacts with the cytoskeleton, GPA, and a multitude of other membrane proteins to build a dynamic cellular model of Band 3 in action.

Key Points

- The structure of the membrane domain of human Band 3 has revealed the molecular details of the substrate-binding site and possible mechanisms of anion transport.
- The structure of human Band 3 has allowed localization of disease-causing mutations and determination of their effect on protein folding and function.
- Models of related SLC4 and 26 transport proteins can be built using the structure of human Band 3 as a template.
- The structure of intact Band 3 still needs to be determined in order to establish the relationship between the cytoplasmic and membrane domains.
- Band 3 does not operate in isolation but as part of a multi-protein complex integral to the integrity and function of the red blood cell.

Figure Legends

Figure 1. Structure of dimeric of mdAE1. (a-b) Cartoon representation of mdAE1 (PDB ID: 4YZF) viewed along the plane of the membrane, with H_2DIDS removed from the model. The 14 TM helices are coloured from N-terminus (blue) to C-terminus (red). The dimerization interface is indicated by asterisks, the anionic substrate by a purple sphere, and substrate passage within subunit B with a grey arrow.

Figure 2. Structure and topology of monomeric mdAE1 (a) The core domain comprises TM1 to 4 from repeat 1 and TM8 to 11 from repeat 2, which can be superposed on top of each other, while the gate domain is made up of TM5 to 7 from repeat 1 and TM12 to 14 from repeat 2, which also superpose well. The TMs and repeats are normally intertwined, but in this figure they are separated for clarity and the pseudo-symmetry rotation axis is shown (gray circle and arrow). (b) Topology diagram of mdAE1 coloured as in (a). mdAE1 contains a single N-linked glycosylation site at Asn642. (c) Close-up view of mdAE1 viewed from the extracellular space showing the core and gate domains, with K539 and K851, from TM5 and 13 respectively, and the crosslinking H_2DIDS represented as sticks.

Figure 3. Key residues in the substrate binding site and localization of some disease-causing mutations. (a) Close-up view of the mdAE1 substrate binding site formed at the N-termini of TM3 and TM10, which meet in the center of the protein near the middle of the membrane. R730 interacts with one of the sulfates (*) from H_2DIDS. (b) Cartoon loop representation of mdAE1 in the same orientation as Fig. 2c. Residues mutated in Hereditary Stomatocytosis that induce a cation leak in mdAE1 are represented by pink spheres. Most of the mutations cluster around the interface of the core and gate domains of the protein, including within TM10 close to the substrate-binding site.

Figure 4. The mdAE1 structure provides additional context for Band 3 function. (a) Cross-section of mdAE1 subunit A (wheat surface colour with grey interior). R730 lies at the vertex of the V-shaped substrate-binding cleft that is open to the extracellular environment. (b) Schematic of possible membrane transport mechanisms that allow the molecule to alternate between outward-open and inward-open conformations to provide the substrate (purple sphere) with access to different sides of the membrane. Yellow arrows indicate domain movements. (c) Cartoon representation of mdAE1 subunit A with amphipathic α-helices that lie parallel to the membrane (H1-H6) colored as in Fig. 1a.

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Figure 1

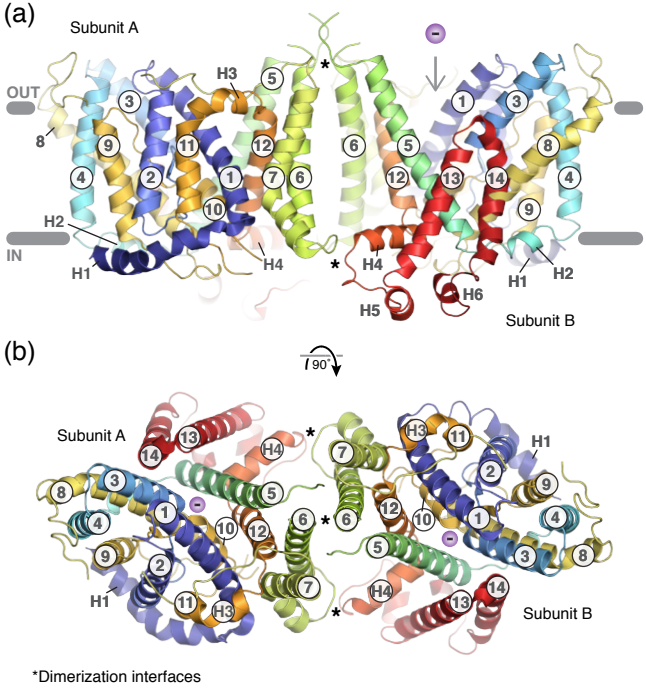

(a)
Subunit A
OUT
IN
H1
H2
H3
H4
H5
H6
Subunit B
(b)
90°
Subunit A
Subunit B
H1
*Dimerization interfaces

Figure 2

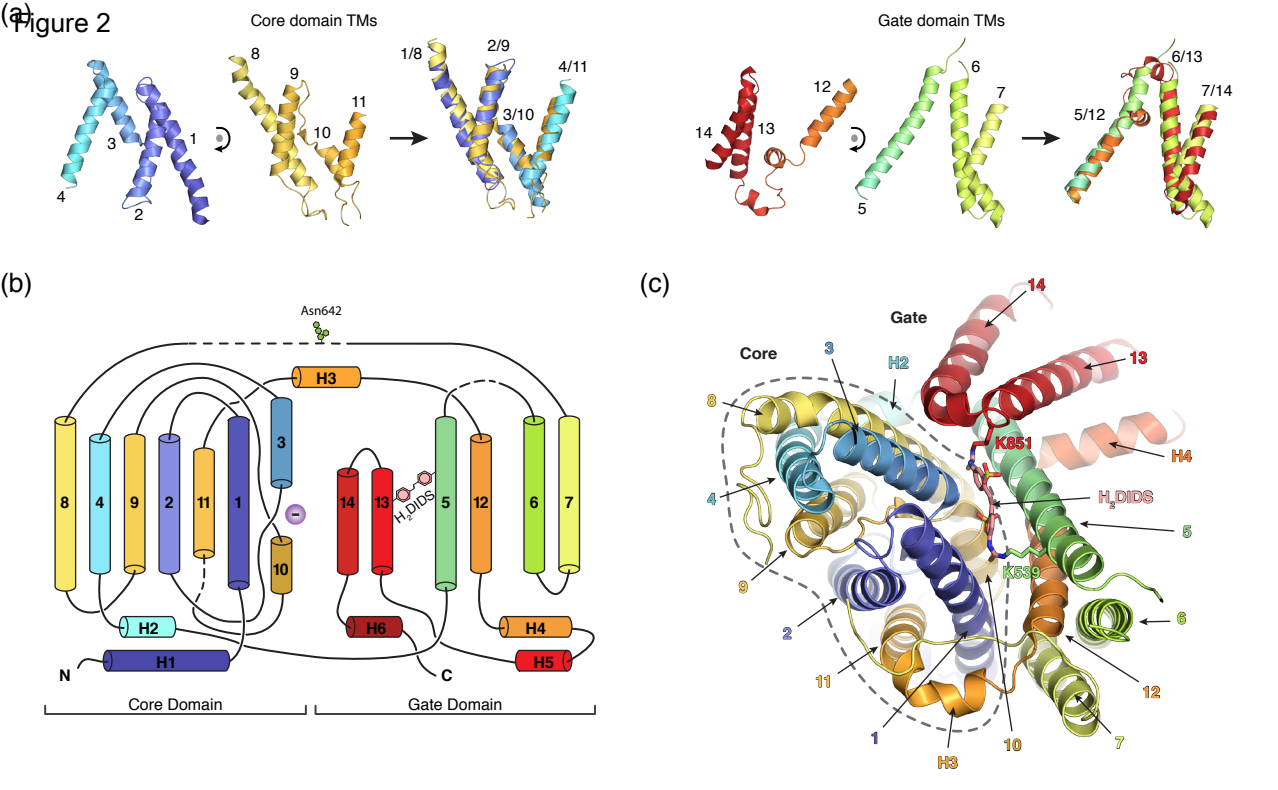

(a)
Core domain TMs
Gate domain TMs
(b)
Asn642
H3
H2
H1
H6
H4
H5
N
C
Core Domain
Gate Domain
H2DIDS
(c)
Core
Gate
H2
H3
H4
H2DIDS
K851
K539

Figure 3

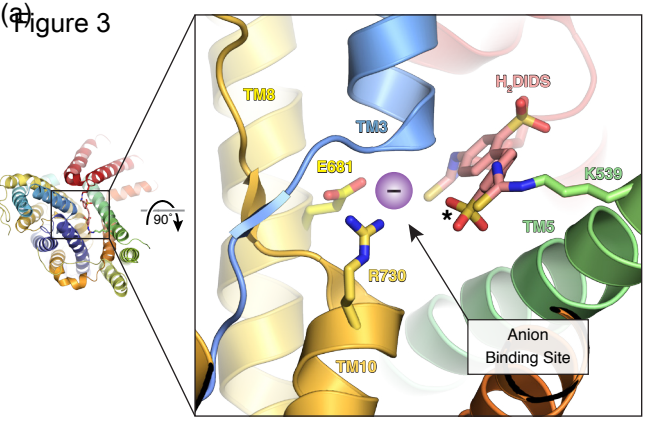
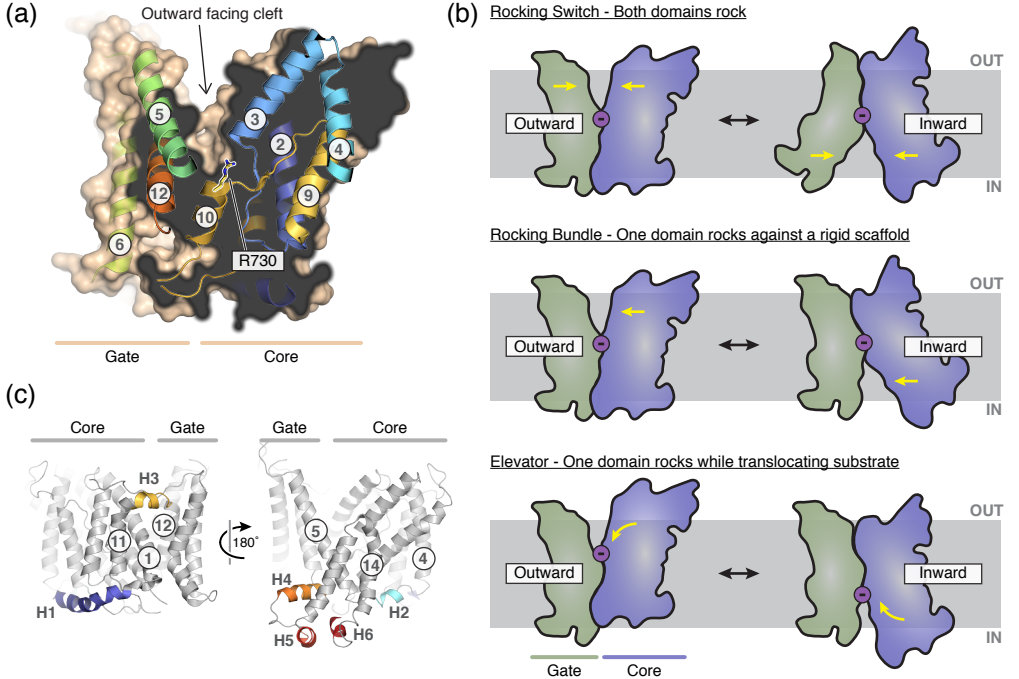

(a)
90°
TM8
TM3
E681
H2DIDS
K539
TM5
R730
TM10
Anion
Binding Site
(b)
Core
L687 (TM8)
G796 (TM12)
(TM9) D705
R730 (TM10)
(TM10-11) E758
(TM11) R760
Gate
S762
(TM11)
S731
(TM10)
H734
(TM10)

Figure 4


(a)
Outward facing cleft
5
3
2
4
12
9
10
6
R730
Gate
Core
(b)
Rocking Switch - Both domains rock
OUT
Outward
Inward
IN
Rocking Bundle - One domain rocks against a rigid scaffold
OUT
Outward
Inward
IN
Elevator - One domain rocks while translocating substrate
OUT
Outward
Inward
IN
Gate
Core
(c)
Core
Gate
Gate
Core
H3
12
11
1
H1
180°
5
14
4
H4
H2
H5
H6