Molecular basis of substrate-induced permeation by an amino acid antiporter

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Transporters of the amino acid, polyamine and organocation (APC) superfamily play essential roles in cell redox balance, cancer, and aminoacidurias. The bacterial L-arginine/agmatine antiporter, AdiC, is the main APC structural paradigm and shares the “5 + 5 inverted repeat” fold found in other families like the Na⁺-coupled neurotransmitter transporters. The available AdiC crystal structures capture two states of its transport cycle: the open-to-out apo and the outward-facing Arg⁺-bound occluded. However, the role of Arg⁺ during the transition between these two states remains unknown. Here, we report the crystal structure at 3.0 Å resolution of an Arg⁺-bound AdiC mutant (N101A) in the open-to-out conformation, completing the picture of the major conformational states during the transport cycle of the 5 + 5 inverted repeat fold-transporters. The N101A structure is an intermediate state between the previous known AdiC conformations. The Arg⁺-guanidinium group in the current structure presents high mobility and delocalization, hampering substrate occlusion and resulting in a low translocation rate. Further analysis supports that proper coordination of this group with residues Asn101 and Trp293 is required to transit to the occluded state, providing the first clues on the molecular mechanism of substrate-induced fit in a 5 + 5 inverted repeat fold-transporter. The pseudosymmetry found between repeats in AdiC, and in all fold-related transporters, restraints the conformational changes, in particular the transmembrane helices rearrangements, which occur during the transport cycle. In AdiC these movements take place away from the dimer interface, explaining the independent functioning of each subunit.

APC transporter | amino acid/Polyamine Antiporter (APA) family | LeuT-fold | X-ray structure | symmetry

The antiporter AdiC is a virtual proton pump that expels protons in *Escherichia coli* and other enteric bacteria in extremely acid environments (1, 2) by exchanging extracellular L-arginine (Arg⁺) for intracellular agmatine (Agm²⁺) (3, 4). AdiC is a member of the basic Amino acid/Polyamine Antiporter (APA) family within the amino acid, Polyamine, and organic Cation (APC) superfamily of transporters (5). The APC superfamily includes two families of eukaryotic transporters that have an impact on human health: Cationic Amino acid Transporters (CATs) and L-Amino acid Transporters (LATs). CATs play key roles in macrophage activation (6, 7) and proliferation (8). Within the LAT members, mutations in b^{0,+}AT and y⁺LAT1 cause the primary inherited aminoacidurias cystinuria B and lysinuric protein intolerance, respectively (9–11), and xCT (system x_c⁻ transporter) is involved in cell redox balance (12), Kaposi’s sarcoma-associated herpesvirus infection (13, 14), and cocaine relapse (15). Finally, LAT1 is overexpressed in many human tumors, thus providing essential neutral amino acids for mTOR (mammalian Target Of Rapamycin) signaling (16). At present, the crystal structures of AdiC (17–19) and the broad-specificity amino acid

transporter ApcT (20) are the closest structural paradigms for CATs and LATs (<20% amino acid identity). AdiC is a homodimer (3, 4) in which each protomer contains the “5 + 5 inverted repeat” fold (17, 18), initially described in Na⁺-coupled symporters (21–24). Mechanistically, the AdiC structures of the wild-type and the double mutant N22A-L123W represent two conformational states within the transport cycle: the open-to-out apo (PDB 3LRB and 3NCY) (17, 18) and the outward-facing Arg⁺-bound occluded (PDB 3L1L) (19), respectively. However, despite these structures, our knowledge of how AdiC recognizes and subsequently translocates Arg⁺ is limited. To provide a better understanding of the mechanism of the 5 + 5 inverted repeat fold-transporters, we have solved the crystal structure of a substrate-bound AdiC mutant in an open-to-out conformation, representing the initial substrate recognition state. The combination of structural, functional, substrate docking, and molecular dynamics (MD) studies reveals that the proper coordination of the substrate guanidinium group with AdiC residues Trp293 and Asn101 is required to transit to the occluded state. Finally, we provide evidence that the symmetry and architecture of the repeats determine the protein domains that move during substrate translocation.

Results

Functional Relevance of Trp293 and Asn101. To address how AdiC recognizes Arg⁺, we docked this substrate in the open-to-out apo structure of AdiC (PDB 3LRB) (17) (Fig. S1). The two thermodynamically most favorable clusters (94% of all possible rotamers) position the substrate in a similar site and orientation as in the structure of the outward-facing Arg⁺-bound occluded conformation (PDB 3L1L) (19). There are only two residues (Trp293 and Asn101) whose side chains interact with Arg⁺ in the occluded conformation and in our docking model using the open-to-out apo structure. In both conformations, the guanidinium group interacts with the side chain of Trp293 (TM8; i.e., Transmembrane segment 8), through a π -cation interaction, and with the side chain of Asn101 (TM3), to form a hydrogen bond [Fig. S1 and (19)]. This observation suggests that Trp293 and Asn101, two residues that are totally conserved among the

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org. (PDB ID code 3OB6).

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Fig. 2. Arg⁺-bound AdiC-N101A is in the open-to-out conformation. 2Fo-*F*c electron density map (contoured at 1 σ in purple) and Fo-*F*c omit electron density map (contoured at 2 σ in green) of the substrate-binding site (Arg⁺ is depicted with green carbon atoms) (A). Trp202 and Trp293 are separated, a characteristic of the open-to-out conformation. Other bulky residues have been included for density quality assessment. Arg⁺ coordination in the AdiC-N101A structure (B). The Arg⁺ α -amino group is next to the negatively charged end of TM6a helix dipole, and at hydrogen-bond distance of Ile205 carbonyl group (unwound segment of TM6). The α -carboxy group lies in the vicinity of the positively charged end of TM1b helix dipole and participates in a hydrogen bond with Ser26 side chain (unwound segment of TM1). The guanidinium group is ~4.6 Å away from Trp293 (TM8) and its nitrogen atoms are at hydrogen-bond distance of Ala96 carbonyl group (TM3) and Ser357 side chain (TM10). (C) Same view as in (B) of the Arg⁺-bound occluded conformation of AdiC (3L1L) (19). (B and C differ in the position of the Arg⁺ guanidinium group as well as in the unwound segment of TM1 (Ile23 to Ser26), where the carbonyl group of Met24 is now further stabilized by hydrogen bonds with residues Ser26, Gly27 and Val28 (B), thereby preventing the collision with Gly21 shown in c (dashed circle).

to that observed in the occluded structure (Fig. 2 *B* and *C*). Interestingly, the main difference appears in the position of the guanidinium group. Compared to the occluded state (PDB 3L1L), this group is shifted away from Trp293 (TM8), Cys97

(TM3), and the mutated Ala101 (TM3) and lies closer to Ala96 (TM3) and Ser357 (TM10).

The N101A structure reveals new insights about substrate recognition and occlusion in AdiC. In addition to the π -cation interaction between the Arg⁺ guanidinium group and the indole moiety of Trp293, the proper orientation between these two groups is also essential to initiate the transition to the occluded state. As seen in this structure, the absence of the Arg⁺-Asn101 interaction leads to a mislocalization of this guanidinium group. Indeed, MD analysis reveals that the mobility of this guanidinium group in the wild-type AdiC open-to-out conformation (PDB 3LRB) is restricted near Trp293, pivoting slightly back and forward to Ser357 (TM10) (Fig. S4); in contrast, in N101A, this group travels freely between Trp293 and Trp202 (TM6a) (Fig. S5). Thus, Arg⁺ in N101A becomes much less competent to find the effective pose in the binding site (Fig. 2C) required to continue the transport cycle; consequently, the open-to-out Arg⁺-bound conformation observed in the crystal structure is stabilized. The functional outcome is a dramatic decrease in the turnover rate (see V_{MAX} of N101A in Table 1). Remarkably, this functional deficiency can be reversed in the N101D mutant by simply reestablishing the capacity of position 101 to form a hydrogen bond and/or a salt bridge with the guanidinium group (Table 1 and Fig. S2B).

Arg⁺ Stabilizes a Semiocluded State upon Properly Stepping on Trp293. The three open-to-out AdiC structures show notable differences in the position of two totally conserved residues in the APA family: Trp202 (TM6a) and Phe350 (loop TMs 9–10). In contrast to Miller and coworkers' (PDB 3NCY) (18) (Fig. 3A) and the current (Fig. 3B) structures, Shi and coworkers (PDB 3LRB) (17) position these two residues next to each other (possible π - π stacking) and closer to the substrate cavity (Fig. 3C), representing a semiocluded state. The exchange between the open-to-out and the semiocluded states can occur by simple thermal motion as a result of the relatively high mobility of loop

Fig. 3. Proposed mechanism of Arg⁺ recognition and induced fit by AdiC. Periplasmic Arg⁺ is recognized by the apo conformation of AdiC (A; 3NCY) and binds with a similar orientation (B; current structure) as in the Arg⁺-occluded conformation (D; 3L1L). The proper Arg⁺ binding samples the semioccluded state (C; docked Arg⁺ in 3LRB) by stabilizing Trp202 (TM6a) and Phe350 (loop TMs 9–10) interaction. This semioccluded conformation evolves to the occluded state mainly by pivoting TM6a. Transition from the apo (A) to the semioccluded state (C) is defective in mutant N101A. TM segments are numbered in italics.

TMs 9–10, as judged by the structural B factors. Notably, MD analysis shows that the Trp202-Phe350 interaction is stabilized when Arg⁺ is present in the binding site but not when it is absent (Fig. S6). Also, transition to the semioccluded state is prevented in N101A regardless the presence of Arg⁺ (Fig. 3B), thereby suggesting that the effective pose of Arg⁺ in the binding site samples the semioccluded state. We speculate that the stabilization of the semioccluded state upon proper Arg⁺ accommodation facilitates the pivoting of TM6a (aliphatic interaction of Trp202 with the alkyl chain of Arg⁺) and TM10 (hydrogen-bond of the Arg⁺ guanidinium group with Ser357) during the transition to the occluded state (Fig. 3D).

The Architecture of the Repeats Dictates the Conformational Changes During the Transport Cycle. Using the Na⁺-coupled leucine transporter (LeuT), Forrest and coworkers first described that the 5 + 5 inverted repeat topology allows the generation of symmetry-related states along the transport cycle (28). For AdiC, we noted that the superposition of repeat 2 (TMs 6–10) over repeat 1 (TMs 1–5) is the result of an almost pure twofold rotation (e.g., 175.2° rotation and a small translation of 0.02 Å in the N101A structure) around the inverted repeat symmetry axis (hereafter referred to as subunit axis). We exploited this pseudosymmetry to model an open-to-in conformation of AdiC from the N101A open-to-out structure using the subunit axis (Fig. S7). To generate the model, we rotated the TMs along the subunit axis and replaced every TM from the 5 + 5 inverted repeat (TMs 1–10) by the corresponding pseudosymmetric TM (e.g., TM3 in place of TM8 rotated), keeping the position of the subunit axis invariable. The equivalent residues between TMs were those defined by the initial superposition of the repeats. Examination of the model showed that the transition from the outward- to the inward-facing states results in the pivoting of both the bundle (TM 1, 2, 6, and 7) and the hash (TM 3, 4, 8, and 9) domains, while TMs 5 and 10 presented minor changes (Fig. S7). Remarkably, application of the same operation to the open-to-out apo structure of the sodium-hydantoin transporter Mhp1 results in a model that reproduces the crystal structure of the open-to-in apo conformation where only the hash domain moves during this transition (Fig. S8) (23, 25); this observation thus validates our open-to-in AdiC model. Moreover, the same maneuver only pivots the bundle in LeuT, as described

previously (28). Our analysis reveals that the orientation of the symmetrically related TMs with respect to the subunit axis can simply explain the different conformational changes proposed for AdiC, LeuT (28), and Mhp1 (25) during the transition between outward- and inward-facing states.

The subunit axes of the protomers in AdiC have a particular disposition with a little deviation (6°) from the plane of the membrane and perpendicular to the dimer axis (Fig. 1A and B). This setting ensures the independent function of each protomer (i.e., free movement of the rocking bundle and hash domains) with minimal alteration of the dimer interface (Fig. 1C). Indeed, in AdiC (18) and in the related eukaryotic LAT transporter b⁰+AT (29) each protomer transports independently.

Discussion

In the well established alternate access model (30) secondary transporters undergo several conformational states in order to translocate the substrate across the membrane. During this transition, the transporter keeps the substrate accessible to only one side of the membrane at a given time by opening and closing different gates (Fig. 4). The energetics of coupling between substrate binding and transporter conformational changes are best explained by the “induced transition fit” mechanism in analogy to enzymes (31). According to this mechanism, initial recognition between the substrate and a nonoptimal binding site of the transporter in the ground state (open-to-in or open-to-out apo conformations) is required to trigger the transition to a transient state (occluded or fully occluded state), where the binding site is reorganized to attain an optimum fit with the substrate. The energy released from this optimum binding will be used by the transporter to compensate the energy required to reach this transition state, necessary to continue the transport catalysis. The substrate-bound occluded structures of 5 + 5 inverted repeat fold-transporters (19, 21, 23, 26, 27), as well the substrate-bound lactose permease LacY structure (32, 33) are examples of the optimum substrate fit and strongly support the substrate-induced transition fit mechanism. The current AdiC-N101A structure reflects the initial substrate recognition state (Fig. 3B), where the substrate is bound to the outward-facing apo conformation (18). Because we cocrystallized AdiC with a substrate rather than an inhibitor, our structure informs about the role of the Arg⁺ gua-

Fig. 4. Symmetrical states along the alternative access mechanism of transporters with the 5 + 5 inverted repeat fold. Upon substrate (red ellipsoid) binding to the open-to-out apo state, the substrate-bound state (represented by the current AdiC structure) evolves to an occluded state, where two gates (thick and thin) prevent the diffusion of the substrate to either side of the membrane (35). Occlusion of the substrate by a thin gate is a common mechanism in the transport cycle of these transporters, in spite of involving different molecular events, as described for LeuT, vSGLT, Mhp1, BetP, and AdiC (19, 21–24). The inward-facing states are symmetrically related to the outward-facing ones. Transition to the inward-facing states requires a transient fully occluded symmetrical intermediate. In ion-coupled symporters (LeuT, vSGLT, Mhp1, ApcT, and BetP) a free transition between the apo structures (outward- and inward-facing) is required to close the transport cycle. The apo occluded structure of ApcT (20) is close to this state. In antiporters (AdiC and CaiT), the return to the outward-facing states requires the binding and translocation of a new intracellular substrate that will move the transporter back through all the states but in the opposite direction. Protein Data Bank access codes are indicated in parentheses.

nidinium group in triggering the transition from the open-to-out to the outward-facing occluded state. Clearly, the proper coordination or “productive pose” of this group stepping on Trp293 (TM8) and interacting with Asn101 (TM3) and Ser357 (TM10), similar to our Arg⁺ docking model in the open-to-out conformation (Fig. S1) is essential to sample the semiocluded state for subsequent transit to the occluded state (Fig. 3 C and D). The N101A mutation leads to a delocalization of the Arg⁺ guanidinium group around the binding site of the open-to-out conformation, thus reducing the probability to attain this “productive pose.” In addition, the binding energy after optimum Arg⁺-AdiC fitting is decreased by the loss of one hydrogen bond (Fig. 2 B and C) making this transition state energetically more costly. Consequently, the AdiC-N101A V_{MAX} is dramatically reduced while K_M is not greatly altered (Table 1). Thus, the current structure together with the previous AdiC structures (17–19) describe for the first time clues on the molecular mechanism of the substrate-induced transition fit in a 5 + 5 inverted repeat fold-transporter. Indeed, among this class of transporters only CaiT has also been crystallized in the initial substrate recognition state (open-to-in) (26, 27), but not in the transient inward-facing occluded state (34).

The current crystal structure also completes the picture of the major conformational states during the transport cycle of the 5 + 5 inverted repeat fold-transporters (Fig. 4), where an inward-facing state corresponds to each outward-facing state, as suggested previously (35). As found for the crystal structures of Mhp1 apo states (Fig. S8), we propose that the symmetry between the repeats dictates the relationship between the conformations of outward- and inward-facing states in these transporters. In particular, fully occluded states (20, 24) represent the most symmetrical arrangement as they correspond to the edge of the transition between outward- and inward-facing conformations. Indeed, evolution appears to have preserved very tightly the presence of essentially pure pseudo-twofold symmetry transformations within all known structures of this fold, with rotation angle values ranging from 170.6° [Mhp1, open-to-out apo (23)] to 179.9° [BetP, fully occluded (24)] and negligible translations along the rotation axis (0.02 Å in the AdiC-N101A current structure). This intrasubunit symmetry framework strongly constrains the overall conformations adopted by these transporters during the transport cycle. This scenario provides a unifying explanation, straightforward from the architecture of the repeats, for previous divergent interpretations: rocking bundle in LeuT (28), rotation of the hash domain in Mhp1 (25), or rotation of the bundle and the hash in AdiC (Fig. S7).

The same symmetry-based arguments could be applied to hypothesize about the AdiC substrate-binding site from the cytosol. In the outward-facing occluded state the subunit axis passes between Trp293 and Arg⁺, and is located ~2.5 Å more inwards than the Arg⁺ center of mass (Fig. S9A) suggesting the existence of a symmetry-related internal substrate-binding site in the inward-facing occluded conformer of AdiC. Consequently, we also applied the previously described symmetrical operation to the outward-facing Arg⁺-bound occluded structure in order to generate the inward-facing Arg⁺-bound occluded conformation (Fig. S9B). For comparisons of structures and symmetry-generated models, the subunit axes were kept fixed. In this new model, Arg⁺ moves to a newly generated cavity situated ~5 Å closer to the cytoplasm along the translocation pathway. Arg⁺ rotates upside down and Trp293 moves away from the center of the cavity, possibly accompanying the guanidinium group of Arg⁺ during the transition (Fig. S9B). The rotation of Arg⁺ allows the coordination of its α -amino and α -carboxyl groups with the charged ends of TM1a and TM6b dipoles (Fig. 4B), similar to

the coordination with TM6a and TM1b ends in the outward-facing occluded conformation (Fig. S9A) (19). Interestingly, in this internal binding site the Arg⁺ α -amino group sits next to Asn22 (TM1), thus suggesting a hydrogen-bond interaction. This scenario would explain the functional and structural features of the N22A mutant, crystallized in the outward-facing Arg⁺-bound occluded conformation (19). We reasoned that the N22A mutation substantially decreases the affinity of Arg⁺ for the internal site, thereby stabilizing the outward-facing Arg⁺-bound occluded conformation, which results in an apparent increase in the affinity for Arg⁺ (19). Although previously proposed for AdiC (19), the presence of two sequential binding sites for Arg⁺ (external and internal) remains to be probed; however, the simple application of the 5 + 5 inverted repeat symmetry supports this concept. Crystal structures of AdiC bound to Agm²⁺ in the internal binding site are needed to demonstrate this hypothesis. Two mutually excluding substrate-binding sites need not be a universal feature for the 5 + 5 inverted repeat fold-transporters; however, in addition to AdiC, there are other structures where the substrate is also bound away from the intrasubunit symmetry axis [e.g., Mhp1 (23)].

The general applicability of a symmetry-based translocation mechanism in combination with the observed structural plasticity might explain why the 5 + 5 inverted repeat fold is widely shared among secondary transporters, including both antiporters and ion-coupled symporters. The main difference in the transport cycle between these transporter classes is that the former can transit only through a substrate-bound intermediate, whereas the cycling of ion-coupled symporters also requires substrate-free transitions (Fig. 4). Although these transporters share the same functioning principles, further research is required to understand the peculiarities imposed by the specialization of the different types of 5 + 5 inverted repeat fold-transporters.

Methods

Protein expression and purification was prepared as described (4) and with minor modifications for crystallization. Transport activity of all AdiC variants was tested by measuring their L-arginine / L-arginine exchange activity after reconstituting the purified protein into lipid vesicles, as described (4). Binding experiments using ITC were performed as described (4). AdiC-N101A was cocrystallized with 1 mM L-arginine by sitting-drop vapor-diffusion methods after 1–3 d. Diffraction data up to 2.8 Å of a cryocooled crystal in a 32% PEG400 buffer allowed the structural resolution achieved by molecular replacement. A density modification stage was introduced previous to structural refinement, which allowed proper tracing of the molecules except for the mobile loop TMs 9–10 in protomer 1. Docking experiments of Arg⁺ to AdiC were performed using AdiC-N101A structure (present structure) and the open-to-out apo structure (3LRB) as templates. We performed 200 ns MD simulations of the AdiC dimer embedded in a fully solvated dipalmitoylphosphatidylcholine membrane in two conformations, namely open-to-out apo (3LRB) and the open-to-out Arg⁺-bound N101A mutant (current structure), in order to assess substrate mobility with respect the N101A mutation and conformational changes of AdiC residues Trp202 and Phe350 in the absence or presence of Arg⁺. Molecular models of AdiC, LeuT, and Mhp1 using the 5 + 5 inverted repeat symmetry were built using a similar approach as described (28). More details are provided in *SI Text*.

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