Protonation of Glutamate 208 Induces the Release of Agmatine in an Outward-facing Conformation of an Arginine/Agmatine Antiporter

Elia Zomot and Ivet Bahar

From the Department of Computational & Systems Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

JUNE 3, 2011 • VOLUME 286 • NUMBER 22 • 19693

Virulent enteric pathogens have developed several systems that maintain intracellular pH to survive extreme acidic conditions. One such mechanism is the exchange of arginine (Arg<sup>+</sup>) from the extracellular region with its intracellular decarboxylated form, agmatine (Agm<sub>2</sub>−). The net result of this process is the export of a virtual proton from the cytoplasm per antiport cycle. Crystal structures of the arginine/agmatine antiporter from *Escherichia coli*, AdiC, have been recently resolved in both the apo and Arg<sup>+</sup>-bound outward-facing conformations, which permit us to assess for the first time the time-resolved mechanisms of interactions that enable the specific antiporter functionality of AdiC. Using data from ~1 μs of molecular dynamics simulations, we show that the protonation of Glu-208 selectively causes the dissociation and release of Agm<sub>2</sub>−, but not Arg<sup>+</sup>, to the cell exterior. The impact of Glu-208 protonation is transmitted to the substrate binding pocket via the reorientation of Ile-205 carbonyl group at the irregular portion of transmembrane (TM) helix 6. This effect, which takes place only in the subunits where Agm<sub>2</sub>− is released, invites attention to the functional role of the unwound portion of TM helices (TM6 Trp-202–Glu-208 in AdiC) in facilitating substrate translocation, reminiscent of the behavior observed in structurally similar Na<sup>+</sup>-coupled transporters.

Transport proteins are usually classified based on the energy used for transport: primary active transporters rely on light, ATP hydrolysis, or redox reactions; secondary active transporters, or symporters, require the electrochemical gradient of ions across the membrane to power the “uphill” translocation of their substrate; and precursor/product antiporters exchange one molecule with its metabolic product independent of another source of energy (1, 2).

Recently resolved crystal structures showed that several secondary active symporters and precursor/product antiporters that belong to distant families share common structural features, consolidating them into a single structural family. These include the leucine transporter (LeuT) from the neurotransmitter/sodium symporters (NSS) family (3), the galactose transporter from sodium/solute symporters (SSS) (4), the betaine transporter BetP and the carnitine/betaine antiporter CaiT from betaine/choline/carnitine transporters (BCCT) (5–7), the benzyl-hydantoin transporter Mhp1 from nucleobase/cation symport-1 family (8), and the arginine/agmatine antiporter AdiC and the ApcT transporter from the amino acid/polyamine/organocation (APC) family (9, 10). The common architecture shared by these structures, referred to as the LeuT fold for short, is characterized by (i) 10 TM2 helices that form the core of the transporter, (ii) an inverted pseudosymmetry whereby the first five TM helices can be superposed on the second by rigid-body rotation of ~180°, and (iii) a disruption in the helical backbone structure around halfway across the membrane in the first helix of each five-helix repeat (TM1 and TM6 in LeuT and in AdiC). Backbone carbonyl and amine groups unable to form hydrogen bonds at these broken α-helical regions exhibit an enhanced disposition/avidity for interacting with the substrate and co-transported sodium ions.

The APC family members carry out the uniport, symport, or antiport of a broad range of substrates across the membrane bilayer (11). Some allow enteric pathogens such as certain strains of *Escherichia coli* and *Salmonella enterica* to survive under extreme acidic conditions where pH can be as low as 1.5–2 (12–15). Such resistance mechanisms are imparted by exchanging extracellular (EC) amino acids with their intracellular (IC) decarboxylated products: AdiC exchanges arginine/agmatine (Arg<sup>+</sup>/Agm<sub>2</sub>−); GadC exchanges glutamate/γ-aminobutyric acid (16, 17); and CadB exchanges lysine/cadaverine (18, 19). A net efflux of one virtual proton per antiport cycle is achieved by AdiC upon exchanging IC Agm<sub>2</sub>− for EC Arg<sup>+</sup> (14, 15).

The structures of two APC members have been resolved to date: the broad specificity amino acid transporter (ApC'T) in an inward-facing, apo state (10) and AdiC in outward-facing apo (open) (9, 20) and Arg<sup>+</sup>-bound (occluded) (21) states. We report here our findings based on molecular dynamics simulations of both AdiC structures (9, 21).

*This work was supported by National Institutes of Health Grants 1R01GM086238-01 and 1U54GM087519-01. This work was also supported by the National Science Foundation through TeraGrid resources provided by Kraken (National Institute for Computational Sciences) and Ranger (Texas Advanced Computing Center) under Grant TG-MCB100108. The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S6, Figs. S1–S7, and Movies M1 and M2.*

<sup>1</sup>To whom correspondence should be addressed: Dept. of Computational & Systems Biology, School of Medicine, University of Pittsburgh, 3064 BST3, 3501 Fifth Ave., Pittsburgh, PA 15213. Tel.: 412-648-3332; Fax: 412-648-3163; E-mail: bahar@pitt.edu.

<sup>2</sup>The abbreviations used are: TM, transmembrane; TMD, TM domain; EC, extracellular; IC, intracellular; r.m.s.d., root mean square deviation.
Protonation of Glu-208 Induces Release of Agmatine

As illustrated in Fig. 1, the apo- and Arg$^+$-bound AdiC structures are highly superposable. The main differences reside in the reorientation of the EC half of TM6 (TM6a); the slight displacements in the EC parts of TM1, TM2, and TM10; and the side chain rotameric states of substrate-coordinating residues near the center of each monomer. The substrate-binding pocket is lined by TM1, TM3, TM6, TM8, and TM10. Trp-202 (on TM6) forms an external layer (EC gate) together with Ser-26; Trp-293 (TM8) forms a middle one; and access to the IC region is blocked by three hydrogen-bonded residues, Tyr-93 (TM3), Glu-208 (TM6), and Tyr-365 (TM10), which presumably serve as the IC gate.

Purified AdiC reconstituted into liposomes has been shown previously to have increased function at pH 4 compared with pH 6, indicating a direct effect of pH on AdiC activity (22). On the EC periphery of AdiC, there are six acidic residues whereas within the TM domain there is only one such residue, Glu-208. Because any impact of the acidity of the external medium on the function of AdiC is more likely to be via modification of the EC periphery of AdiC, there are six acidic residues whereas within the TM domain there is only one such residue, Glu-208. Because any impact of the acidity of the external medium on the function of AdiC is more likely to be via modification of the protonation state of residues within the TM domain than on the peripheral loops, we decided to focus on the functional effect of protonation in Glu-208, the only amino acid within this domain that may possibly be affected by external decrease in pH.

The p$K_a$ of Glu-208 is predicted by PropKa (23, 24) to be 6.4 and 6.1 in the respective open and occluded conformations, whereas the H++ server (Virginia Tech) yields respective p$K_a$ values of 3.0 and 2.9. In addition to this inconsistency in the computational estimation of the p$K_a$ of Glu-208, it is problematic to investigate experimentally the effect of lowering pH below 4 on AdiC mechanisms. However, molecular dynamics simulations may provide a direct means of looking into the effect of different protonation states of Glu-208 in substrate binding properties and AdiC dynamics. In this study, we present the first molecular dynamics insight into the time-resolved interactions at the binding pocket of AdiC and show that protonation of Glu-208 located ∼8 Å away from the substrate toward the cytoplasm prompts the release of Agm$^{2-}$, but not Arg$^+$, into the EC region. This step is facilitated by backbone rearrangement in the unwound part of TM6 at Ile-205, the carbonyl group of which participates in binding the substrate amino group.

EXPERIMENTAL PROCEDURES

Materials—The crystal structures of the apo- and Arg$^+$-bound AdiC (Protein Data Bank codes 3LRB and 3L1L, respectively) were used in our molecular dynamics studies. Dimeric forms were simulated because of their physiological relevance (22) and to achieve better statistical sampling of functional events at the binding sites. Residues 1–5 (N terminus), 253–272 (fourth EC loop, EL4), and 436–445 (C terminus) were missing in the apo form; whereas residues 1–6, 181–191, and 441–445 were missing in the Arg$^+$-bound form. Residues 181–191 of the latter structure were reconstructed as a loop using the Sybyl software (version 8.0, Tripos International, St. Louis, MO), given that this segment overlapped with the mainly disordered portion of the long EC-exposed loop EL3 (residues 173–187; connecting TM5 and TM6), resolved in the apo form. The EL4 in the apo form was reconstructed upon superposition onto the Arg$^+$-bound form using UCSF Chimera (25). Atomic coordinates for the residues that had crystallographically resolved Cα coordinates but one or more missing atoms were completed, along with all hydrogen atoms, using the AutoPSF plugin of VMD (version 1.8.7) (26).

Setups—To achieve optimal comparison between the dynamics of the apo/open and substrate-bound/occluded forms of the outward-facing AdiC, we adopted a series of similar simulation setups and identical equilibration and production run protocols with different starting conformers, summing up to a total duration of 0.92 μs. The properties of these conformers, which apply to both subunits in every setup, are listed in Table 1. Simulations were repeated for the protonated and deprotonated states of Glu-208, in the presence or absence of Arg$^+$, Agm$^{2-}$, or Arg$^{2+}$. Arg$^{2+}$, the dominant state of arginine at pH < 2, is identical to Arg$^+$ except for the protonation of the backbone carboxylate group.

Protocols—Each complete AdiC dimer was initially placed in a 1-palmitoyl-2-oleoyl-sn-glyero-3-phosphatidylethanolamine membrane bilayer of dimensions 140 × 100 × 50 Å$^3$ and fully solvated. Lipid molecules within 2 Å from the protein were removed, and 0.5-ns simulations were performed, during which all protein and substrate atoms were fixed to ensure that the lipid molecules were optimally packed around the protein. The protein and lipid membrane were then re-solvated in 150 mM NaCl, and the system, composed of ∼$10^6$ atoms (supplemental Table S14), was energy-minimized and equilibrated for a total of 2.5 ns at 310 K (0.5 ns with the AdiC backbone constrained, followed by 2 ns of constraint-free simulation). At the end of the equilibration phase, changes specific to various setups (Table 1) were made. These changes were as follows: 1) the conversion of N22A (occluded structure) to WT and 2) protonation of Glu-208 and/or replacement of Arg$^+$ by Arg$^{2+}$/Agm$^{2+}$. Each system was then minimized for another 20,000 steps before initiating the production runs. All simulations were performed using the NAMD software (version 2.7b2) (27), with time steps of 1 fs and 2 fs in the respective equilibration and production run phases. To provide a more accurate measure for the stability of the TM domains that include the functional core of AdiC, we report the root mean square deviations (r.m.s.d. values) in atomic coordinates for both the “full” transporter, and the core composed of TM helices and the stable structural elements in the EC and IC regions, called the extended TM domain (eTMD). The eTMD excludes the amino acids 1–12 and 429–445 at the N and C termini, and the residues 173–189 and 251–272 in the two longest EC loops, EL3 and EL4, which have been reconstructed in Arg$^+$-bound and apo forms, respectively. The r.m.s.d. of the eTMD provides a better metric for assessing the stability of the antipporter structure.

RESULTS

Intrinsic Tendency of Apo/Open AdiC to Reconfigure toward Substrate-bound/Closed State Even in Absence of Substrate—The crystallized apo conformation, where the EC aqueous cavity is more open, differs from the Arg$^+$-bound form mainly by a rotation of ∼40° in TM6a, along with a slight displacement in the EC parts of TM2 and TM10 away from the core region.
Protonation of Glu-208 Induces Release of Agmatine

The isomerization of Trp-202 is accompanied by changes in interatomic distances near the substrate-binding pocket. In particular, Ser-26 Oγ and Trp-202 Ne6, separated by ∼10Å in the apo/open structure, come into close proximity similar to their separation in the substrate-bound occluded form (Fig. 2A). Likewise, Ile-205 and Trp-202, originally located at the disordered region on TM6, restore their broken hydrogen bonds, consistent with their interaction in the substrate-bound form (supplemental Table S3). Finally, the distance between the

(FIG. 1). The two structures align with an r.m.s.d. of 2.54 Å upon exclusion of the relatively mobile N- and C-terminal segments and the EC loops EL3 and EL4. The superposed eTMDs are highly stable: the r.m.s.d. of the equilibrated structures remains less than 2.5 Å in all runs (Table 1 and supplemental Table S1B and Fig. S1). This is despite the fact that many EC water molecules penetrated deep enough into the binding pocket to interact directly with eTMD core residues, including Glu-208 (set-ups 1a and 1b) (Fig. 2B).

In the Arg⁺-bound form, Trp-202 forms the main gate, shielding the substrate from the EC medium. This external gate is open in the apo form (Fig. 1). Yet, in each of the two independent runs performed for the apo dimer, without and with protonation of Glu-208 (set-ups 1a and 1b, respectively), one of the subunits exhibited a conformational switch to assume a relatively closed state geometrically similar to that observed in the Arg⁺-bound form (Fig. 2A and supplemental Fig. S2); this switch occurred as early as the equilibration step in each case and was maintained throughout the two 60-ns runs. Essentially, the backbone dihedral angle ψ and the side chain dihedrals χ₁ and χ₂ of Trp-202 in subunit B underwent rotameric jumps from their respective original values of −8°, 93°, and 131° to around −30°, ± 180°, and 68° at the onset of both runs, and practically maintained these new angles within ± 20° fluctuations, approximately, throughout the entire duration of the simulations (Fig. 3, A and B). These three values are typical of the isomeric state of Trp-202 in the closed (substrate-bound) conformer (see supplemental Table S2A). Comparison of the fluctuation profiles for the Trp-202 φ, ψ, χ₁, and χ₂ angles in subunit B of the apo antiporter (Fig. 3, A and B) with their counterparts in the ligand-bound forms (Fig. 3, C and D, and supplemental Fig. S3) shows that subunit B essentially assumes the same isomeric state (corresponding to the closed form of the EC gate) as that observed in the ligand-bound structures; and this property is repeated in both runs 1a and 1b. Trp-202 in subunit A, on the other hand, exhibits an alternative rotameric state, distinguished by a χ₂ dihedral angle that departs from that assumed in the original apo state and that stabilized in the occluded state. Notably, both rotamers of Trp-202 were stable, and no interconversions were observed throughout the entire duration of simulations.

The isomerization of Trp-202 is accompanied by changes in interatomic distances near the substrate-binding pocket. In particular, Ser-26 Oγ and Trp-202 Ne6, separated by ∼10Å in the apo/open structure, come into close proximity similar to their separation in the substrate-bound occluded form (Fig. 2A). Likewise, Ile-205 and Trp-202, originally located at the disordered region on TM6, restore their broken hydrogen bonds, consistent with their interaction in the substrate-bound form (supplemental Table S3). Finally, the distance between the
Protonation of Glu-208 Induces Release of Agmatine

Trp-202 and Met-104 (TM3) Cα atoms is decreased by ~3.0 Å, to approximate that in the substrate-bound structure (supplemental Fig. S2). Overall, there is an approach between the EC ends of TM1-TM6 and TM6-TM3 pairs, which is irrespective of the protonation state of Glu-208. The microscopic motions observed in the molecular dynamics runs of the open/apo state thus tend to bring the three-dimensional rearrangements of the residues that line the binding site closer to those seen in the occluded, substrate-bound form. These observations support the predisposition of the apo form to accommodate the bound substrate.

In contrast to Trp-202, which mediates the EC gate, the residues whose side chains potentially serve as the intermediate (Trp-293) and IC (Tyr-93, Glu-208, and Tyr-365) gates undergo more restricted fluctuations (Fig. 2 and supplemental Fig. S2, right panels). An exception is Glu-208, the side chain rotations of which tend to destabilize the hydrogen bond network formed between Tyr-93, Glu-208, and Tyr-365. Tyr-365 hydroxyl can form instead a stable hydrogen bond with Ile-205 carbonyl oxygen if Glu-208 is protonated (supplemental Table S2); these were observed to change to (~63°, 8°, 93°, and 131°) (see supplemental Table S2); these were observed to change to (~63°, ~30°, ±180°, and 68°) in subunit B at the onset of simulations in both runs and fluctuate around these new values throughout the remainder of the simulations (shown). This new set of dihedral angles is comparable to those assumed in the substrate-bound form (C and D).

Substrate Coordination Is Highly Stable in Absence of Glu-208 Protonation—Binding of Arg+ and Agm2+ to AdiC have been reported to be enthalpy-driven under physiological pH, with $K_d$ values of ~100 and ~30 μM, respectively (22, 28). The Arg+–bound crystal of AdiC was obtained for the N22A mutant, which showed uptake levels similar to the WT but higher affinity for binding Arg+ (by ~6-fold) compared with WT AdiC (21). We mutated AdiC-N22A back to WT in silico as the lower affinity for Arg+, and perhaps Agm2+, might help us observe unbinding events within the time frame of our simulations.

Simulations performed for Arg+–bound occluded AdiC in the absence of Glu-208 protonation (setups 2a and 2b), under the conditions where AdiC was crystallized (21), show that the geometry of the binding pocket is conserved over the entire durations of simulations: Arg+ remains positioned between the outer and middle layers formed by the Trp-202 (TM6) and Trp-293 (TM8) side chains, respectively (Fig. 4A); its guanidinium group is coordinated by the carbonyl groups of Ala-96 and Cys-97 and the side chain of Asn-101; its amino end by the carbonyls of Ile-23 (TM1) and Trp-202 and Ile-205 (TM6); and its carboxyl end by the Ser-26 hydroxyl group and the Ser-26 and Gly-27 amides at the disordered segment on TM1. Also, the interactions of Glu-208 with Tyr-93 and Tyr-365 (IC gate)
Glu-208 and the substrate, Arg\(^{+}\), Arg\(^{2+}\), or Agm\(^{2+}\) (supplementary Table S5, last 2 columns). Despite this destabilizing effect, the Arg\(^{+}\) - and Arg\(^{2+}\)-bound forms exhibited dynamic trends closely reproducing those observed for Arg\(^{+}\) - and Agm\(^{2+}\)-bound AdiC in the absence of protonation: Arg\(^{+}\) practically maintained its crystal structure binding geometry over the entire trajectories; its amino end remained coordinated by the carbonyl groups of Trp-202 and Ile-205, its carboxylic end by Ser-26 (OH) and Gly-27 (NH), and its guanidinium group by the carbonyls of Ala-96, Cys-97, and the side chain of Asn-101 (supplemental Fig. S4A). Similar properties hold for Arg\(^{2+}\)-bound Glu-208(H)-AdiC in setups 5a and 5b (supplemental Fig. S4B).

The simulations of the Agm\(^{2+}\)-bound AdiC with protonated Glu-208, on the other hand, displayed a strikingly different picture. The destabilization of the hydrogen bond pattern at the IC gate induced in this case a rotation, within the first 10 ns, in the Ile-205 carbonyl group by up to ~180° toward the internal gate residues Tyr-93, Glu-208, and Tyr-365. This rotational transition was observed in two out of the four simulated subunits (subunits A and B in the respective runs 6a and 6b (Fig. 5 and supplemental Fig. S5). Ile-205 lies on the unwound part of TM6 below Trp-202. Upon rotation, the Ile-205 carboxyl, which initially formed a hydrogen bond with the Agm\(^{2+}\) amine group, interacted instead with the protonated Glu-208 (COOH) and/or Tyr-365 (OH). Fig. 6 and supplemental Fig. S5 illustrate the results, consistently reproduced in two pairs of independent runs (3a, 3b, 6a, and 6b). One effect of this step was the abolishment of the attraction between Agm\(^{2+}\) and Ile-205. Another was the complete dissociation of Agm\(^{2+}\) from the binding pocket in these two subunits into the EC medium, as described below.

Solvation of Binding Site Plays a Key Role in Driving Substrate Translocation to Completion—It is important to note that the Ile-205 backbone conformational switch together with the release of the substrate (Agm\(^{2+}\)) was not observed in any of the trajectories generated for deprotonated Glu-208, AdiC (runs 2 and 3). Among the simulations performed in the presence of Glu-208 protonation, one subunit with Arg\(^{2+}\)-bound AdiC also exhibited such a conformational switch (subunit A in setup 5a). However, Arg\(^{2+}\) was not released in this case. In all production runs of the “occluded” conformation (runs 2–6), the substrate-binding site was not completely inaccessible to the EC medium at all times: up to five water molecules could enter the confines of the binding pocket and interact with the substrate; and the magnitude of these interactions was greatest in the two cases where Agm\(^{2+}\) was released (supplemental Table S5), inviting attention to the role of solvation in facilitating substrate release.

Translocation Pathway of Agmatine to Reach EC Region from Its Binding Pocket—In subunits where the substrate interaction with Glu-208 was lost and the Ile-205 conformational switch took place, complete dissociation of the substrate (Agm\(^{2+}\)) from the binding pocket and into the EC medium took place. This was seen in two cases, which had similar pattern and pathway of unbinding: subunit A in setup 6a, starting at ~80 ns (Fig. 7), and subunit B in setup 6b, at ~23 ns (supplemental Fig. S6). The corresponding animations may be found in supplemental Movie M2, A and B.

**Protonation of Glu-208 Induces Release of Agmatine**

**FIGURE 4. Binding geometries of Arg\(^{+}\) and Agm\(^{2+}\) in the absence of Glu-208 protonation observed at the end of 100-ns simulations.** A and B illustrate the binding pose of the two respective substrates viewed from the EC side at the end of the respective runs 2a and 3a (see Table 1). The binding pocket of subunit A is shown in both cases. Gating residues are colored according to the respective TM helices as described in the legend to Fig. 1 and residues that make interatomic contacts of <2.5 Å from the substrate are in Corey-Pauling-Koltun coloring. Similar geometries were seen in two independent sets of simulations (i.e., runs 2b and 3b; see Table 1), except for Agm\(^{2+}\) in subunit B in run 3a (Fig. 5). The same stable binding pose shown here was selected by Arg\(^{+}\) and Agm\(^{2+}\) in the presence of Glu-208 protonation by the end of the respective runs 4a–b and 5a–b (see supplemental Fig. S4).

**FIGURE 5. Mobility of Agm\(^{2+}\) and its potential interaction with Glu-208\(^{–}\).** Shown here is the pose of Agm\(^{2+}\) in subunit B viewed from two different angles (side views) through the plane of the membrane. Snapshots taken from run 3a at 0 ns and at 100 ns (lighter and darker colors, respectively) show the dislocation of Agm\(^{2+}\) to interact with Glu-208.

are maintained throughout the entire production runs in both subunits (supplemental Table S4 and Movie M1).

The same overall position of the substrate in the binding site, i.e. the confinement between the Trp-202 and Trp-293 side chains that lie parallel to each other, and coordination of terminal charged groups by mostly backbone polar groups on the surrounding helices TM1, TM6, and TM8, was also seen when Arg\(^{+}\) was replaced by Agm\(^{2+}\) (runs 3a and 3b; Fig. 4B). In the latter case, stronger interactions were seen between Agm\(^{2+}\) and Glu-208 due to the lack of a negatively charged carboxylic group and the greater mobility of Agm\(^{2+}\) to optimize its interactions. In one case, Agm\(^{2+}\) was able to shift and directly interact with Glu-208 via its guanidinium group (subunit B, run 3b, shown in Fig. 5). This type of close interaction with Glu-208 accessible to Agm\(^{2+}\), but not Arg\(^{+}\), already signals that the protonation of Glu-208 may have significant consequences on the positioning of Agm\(^{2+}\) within its binding pocket and on dislodging Agm\(^{2+}\) to trigger its release to the EC medium, as described next.

**Glu-208 Protonation Weakens Interaction with Substrate and Triggers Release of Agm\(^{2+}\)**—Similarly to the open apo state, we repeated simulations for the substrate-bound occluded form with protonated Glu-208. These were performed with Arg\(^{+}\), Arg\(^{2+}\), and Agm\(^{2+}\) (respective runs 4–6).

The most prominent consequence of the protonation of Glu-208 was the abolishment of the attractive interaction between
The sequence of events leading to the release of Agm$_2$$^+$ exhibited close similarities between the two independent runs: first, the amino end of Agm$_2$$^+$ dissociated from the binding site passing between Trp-202 and Ser-26 and leading the translocation toward the EC region. In contrast to expectation, this step was not accompanied by noticeable change in the geometry of the backbone or side chain of Trp-202 (Fig. 3 and supplemental Fig. S6). However, a greater tendency for Ser-26 and Trp-202 to move farther from each was seen in subunits where Agm$_2$$^+$ was bound to AdiC with Glu-208 protonated (supplemental Table S4). The Agm$_2$$^+$ amino group translocation across Trp-202 and Ser-26 varied in duration from $\sim$5 ns in setup 6a to $\sim$25 ns in setup 6b. This step was followed by the movement of Agm$_2$$^+$ along Ser-203 and Val-199 on TM6, Gly-27 and Leu-30 on TM1, and subsequently Ile-107 on TM3. Afterward, the Agm$_2$$^+$ molecule in both cases reached and resided for at least 10–15 ns at the external face of AdiC within a space confined by the side chains of Glu-349 on TM10, Glu-409 on TM12, and Ser-195 and Asn-198 on TM6 (Fig. 7 and supplemental Fig. S6). No detectable changes in structure were seen within the 10–15 ns that succeeded the complete dissociation of Agm$_2$$^+$.

Conformational Switch in Ile-205 Can Also Take Place in Open Apo Conformation—In the initial structure of the open apo conformation, both carbonyl groups of Ile-205 face the binding site as in the substrate-bound occluded structure. During the equilibration step used for both runs 1a and 1b, where the major rearrangements mentioned above took place, the carbonyl group of Ile-205 switched its geometry, as in the Agm$_2$$^+$-bound AdiC with Glu-208 protonated, in subunit B but not A. During the production runs (setups 1a and 1b), however, Ile-205 switched back to the original orientation in setup 1a (with Glu-208 deprotonated) after less than 20 ns (supplemental Fig. S7A) but not in setup 1b (with Glu-208 protonated) where it remained stably bound to the hydroxyl of Tyr-365 over the entire trajectory (supplemental Fig. S7B). Thus, the “switch” role played here by Ile-205 is a property of the transporter.
architecture, presumably imparted by the disruption of TM6, which may be prompted irrespective of substrate binding. The stabilization of the new conformational state, however, occurs in the presence of Glu-208 protonation, exclusively.

**DISCUSSION**

In this study, we have investigated the interactions of the substrates at the binding site of AdiC, an antiporter that functions under extreme acidic conditions, which has thus evolved to be structurally and functionally stable notwithstanding protonation of its acidic residues. The above study sheds light to the role of a glutamate (Glu-208) protonation in triggering the translocation of Agm$^2$$^+$ from its binding site to the EC medium. The main observations made in the present study can be summarized as follows.

1) In the apo form, Trp-202 has a tendency for closing on the binding site and adopting a stable geometry that is similar to that of the substrate-bound, occluded conformation. This tendency is evidenced by the changes in dihedral angles and inter-residue distances observed at the equilibration stage, which were maintained throughout the entire duration of long simulations (illustrated in Fig. 3 and supplemental Fig. S3, and listed in supplemental Tables S2 and S3).

2) A highly stable binding pose is observed for Arg$^+$ in both subunits at the end of four independent simulations, irrespective of the protonation state of Glu-208 (Fig. 4A and supplemental Fig. S4A). The side chains of two tryptophans (Trp-202 on TM6, serving as the EC gate, and Trp-293 on TM8, as the intermediate gate) lying almost parallel to each other confine the substrate in a relatively extended conformation, whereas the three functional groups at the amino acid termini and side chain are coordinated by several polar groups on the backbone and side chains of the surrounding helices TM1, TM3, and TM6. The same binding pose was also found to be reproduced in the simulations with Arg$^2$+ (supplemental Fig. S4B).

3) A different picture is observed in the simulations with Agm$^2$$^+$. Although the Arg$^+$ (or Arg$^2$+) binding pose is observed to be a stable binding pose for Agm$^2$$^+$ as well (Fig. 4B), Agm$^2$$^+$ enjoys a higher conformational freedom due to its lack of terminal carboxylic group, it tends to allow for increased entry of water molecules and solvation of the substrate binding site compared with Arg$^+$ (supplemental Table S5); and it may even be dislodged to closely interact with Glu-208 (Fig. 5). This increased mobility and potential interaction with Glu-208 renders it sensitive to the protonation state of Glu-208.

4) Glu-208 protonation has two effects on the binding/unbinding properties of the substrate, one direct and the other indirect. The direct effect is the abolishment of the attractive electrostatic interaction between Glu-208$^-$ (prior to protonation) and the positively charged substrate. This effect is common to both Arg$^+$ and Agm$^2$$^+$, although the latter is more pronounced (supplemental Table S5) due to the double charge of Agm$^2$$^+$. The indirect effect is the disruption of the favorable (attractive) interaction between the substrate and Ile-205 carbonyl group, due to the reorientation of the latter to interact (to form a hydrogen bond) with the protonated carboxylate group of Glu-208 and with the hydroxyl group of Tyr-365 (Fig. 6 and supplemental Fig. S5). Note that this indirect effect is facilitated by the predisposition of Ile-205 to assume such alternative conformations, as a consequence of the disruption of TM6 helical hydrogen bond pattern at this particular region. This property is also evidenced by the apo/open AdiC structure and dynamics (supplemental Fig. S7).

5) The above two effects of Glu-208 protonation, also complemented by a destabilizing effect of EC water molecules that...
inundate the Agm\(^{2+}\) binding pocket (significantly more efficiently than the Arg\(^{+}\) binding pocket), precipitate the dissociation and rapid translocation of Agm\(^{2+}\) to the EC medium (Fig. 6 and supplemental movies M2, A and B). Among other local rearrangements during this process, we noted the increased separation between the external gate Trp-202 and Ser-26 when Agm\(^{2+}/\)Arg\(^{+}\), but not Arg\(^{+}\), is present in the binding site.

Despite its importance, the ability of AdiC to function under extreme acidic conditions has not been experimentally tested. One reason for this is that it is problematic to investigate liposomes with AdiC purified and reconstituted at pH below 4 (22). Such systems are imperative for the study of the direct impact of pH on AdiC because they exclude the involvement of other cellular components and allow the control of the composition of both the internal and external media. In the absence of these experimental approaches, the present simulations of AdiC with Glu-208 protonated and deprotonated provide first insights about the possible mechanism of substrate export by the antiporter.

Notably, substrate release to the EC medium is only one step of the complete antiport cycle, and the remaining steps, including global conformational changes between outward- and inward-facing conformers, the import of Arg\(^{+}\) to the cytoplasm, and the possible mechanisms and stages of protonation and deprotonation of acidic residues, remain to be clarified. The present study sheds light on the substrate export step only; it examines the process under different conditions (apo/open and occluded/substrate-bound states of AdiC, in the presence of different substrates, and different protonation states of Glu-208) and shows that Glu-208 protonation does not have an effect on Arg\(^{+}\) binding or export to the EC region in the outward-facing conformer of AdiC but facilitates the export of Agm\(^{2+}\), consistent with the function of AdiC.

The crystal structure of the outward-facing open structure suggests that Glu-208 could be accessible to water from the EC medium, and our simulations indicate that these water molecules penetrate deep into the substrate binding site up to Glu-208. If protonation occurs, 1) it should precede Arg\(^{+}\) binding when AdiC is in the outward-facing state, 2) the binding site will have to retain the proton, most probably via Glu-208 protonation, when AdiC shifts to the inward-facing state (otherwise, the release of this proton to the IC would render the antiport pathway in both cases of Agm\(^{2+}\) dissociation from the binding site into the EC suggests possible physiological relevance.

It is interesting to note the glutamate at position 208 is fully conserved, along with a hydrophobic residue at position 205, among the APC members involved in acid resistance such as AdiC, PotE and CadB/C, and GadC. In the other members of the family, a negative charge at position 208 and a hydrophobic residue at 205 are mostly absent. In the absence of experimental data, it is tempting to postulate possible cooperation between these two positions in acid-sensing and substrate efflux in response to pH change among the antiport systems of extreme acid resistance. Replacement of Glu-208 with alanine or even aspartate has indeed been shown by Gao et al. (9) to strongly impact antiporter activity and the possibility that Glu-208 may serve as a pH sensor also has been proposed. We also note that the conformational switch role of Ile-205 is enabled by the spatial proximity of Tyr-365, the hydroxyl group of which serves as an attractor to the carbonyl group of Ile-205. Uptake activity as well as binding affinity of Arg\(^{-}\) and/or Agm\(^{2+}\) are strongly impaired in AdiC mutants where the inner gating residues Tyr-93 or Tyr-365 are replaced by alanine (9), which supports the functional significance of Tyr-365 observed in the present study.

On the level of the overall antiport cycle, one implication of the observed unbinding of Agm\(^{2+}\) is that, in the outward-facing occluded state, Agm\(^{2+}\) may be able to dissociate into the EC medium prior to the antiporter shifting to an open state similar to the crystallized structure and the binding (and influx) of Arg\(^{+}\). The time scale of such a global change in conformation would be of the order of micro- to milliseconds.

Acknowledgment—We acknowledge the assistance of Yaakoub El Khamra.
REFERENCES
1. Law, C. J., Maloney, P. C., and Wang, D. N. (2008) *Annu. Rev. Microbiol.* 62, 289–305
2. Poolman, B. (1990) *Mol. Microbiol.* 4, 1629–1636
3. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) *Nature* 437, 215–223
4. Faham, S., Watanabe, A., Besserer, G. M., Cascio, D., Specht, A., Hirayama, B. A., Wright, E. M., and Abramson, J. (2008) *Science* 321, 810–814
5. Ressl, S., Terwisscha van Scheltinga, A. C., Vonrhein, C., Ott, V., and Ziegler, C. (2009) *Nature* 458, 47–52
6. Schulze, S., Köster, S., Geldmacher, U., Terwisscha van Scheltinga, A. C., and Kühlbrandt, W. (2010) *Nature* 467, 233–236
7. Tang, L., Bai, L., Wang, W. H., and Jiang, T. (2010) *Nat. Struct. Mol. Biol.* 17, 492–496
8. Weyand, S., Shimamura, T., Yajima, S., Suzuki, S., Mirza, O., Krusong, K., Carpenter, E. P., Rutherford, N. G., Hadden, J. M., O’Reilly, J., Ma, P., Saidijam, M., Patching, S. G., Hope, R. J., Norbertczak, H. T., Roach, P. C., Iwata, S., Henderson, P. J., and Cameron, A. D. (2008) *Science* 322, 709–713
9. Gao, X., Lu, F., Zhou, L., Dang, S., Sun, L., Li, X., Wang, J., and Shi, Y. (2009) *Science* 324, 1565–1568
10. Shaffer, P. L., Goehring, A., Shankaranarayanan, A., and Gouaux, E. (2009) *Science* 325, 1010–1014
11. Jack, D. L., Paulsen, I. T., and Saier, M. H. (2000) *Microbiology* 146, 1797–1814
12. Audia, J. P., Webb, C. C., and Foster, J. W. (2001) *Int. J. Med. Microbiol.* 291, 97–106
13. Foster, J. W., Park, Y. K., Bang, I. S., Karem, K., Betts, H., Hall, H. K., and Shaw, E. (1994) *Microbiology* 140, 341–352
14. Gong, S., Richard, H., and Foster, J. W. (2003) *J. Bacteriol.* 185, 4402–4409
15. Iyer, R., Williams, C., and Miller, C. (2003) *J. Bacteriol.* 185, 6556–6561
16. Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F., and Foster, J. W. (1999) *J. Bacteriol.* 181, 3525–3535
17. Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L., and Slonczewski, J. L. (1996) *J. Bacteriol.* 178, 3978–3981
18. Soksavatmaekhin, W., Kuraishi, A., Sakata, K., Kashiwagi, K., and Igarashi, K. (2004) *Mol. Microbiol.* 51, 1401–1412
19. Soksavatmaekhin, W., Uemura, T., Fukikawa, N., Kashiwagi, K., and Igarashi, K. (2006) *J. Biol. Chem.* 281, 29213–29220
20. Fang, Y., Jayaram, H., Shane, T., Kolmakova-Partensky, L., Wu, F., Williams, C., Xiong, Y., and Miller, C. (2009) *Nature* 460, 1040–1043
21. Gao, X., Zhou, L., Jiao, X., Lu, F., Yan, C., Zeng, X., Wang, J., and Shi, Y. (2010) *Nature* 463, 828–832
22. Fang, Y., Kolmakova-Partensky, L., and Miller, C. (2007) *J. Biol. Chem.* 282, 176–182
23. Li, H., Robertson, A. D., and Jensen, J. H. (2005) *Proteins* 61, 704–721
24. Bas, D. C., Rogers, D. M., and Jensen, J. H. (2008) *Proteins* 73, 765–783
25. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) *J. Comput. Chem.* 25, 1605–1612
26. Humphrey, W., Dalke, A., and Schulten, K. (1996) *J. Mol. Graph.* 14, 33–38
27. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) *J. Comput. Chem.* 26, 1781–1802
28. Casagrande, F., Ratera, M., Schenk, A. D., Chami, M., Valencia, E., Lopez, J. M., Torres, D., Engel, A., Palacin, M., and Fotiadis, D. (2008) *J. Biol. Chem.* 283, 33240–33248