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Protonation States of Ammonia/Ammonium in the Hydrophobic Pore of Ammonia Transporter Protein AmtB

Hiroshi Ishikita*,†,§ and Ernst-Walter Knapp‡

Contribution from the Department of Chemistry, The Pennsylvania State University, 104 Chemistry Building, University Park, Pennsylvania 16802, and Institute of Chemistry and Biochemistry, Free University of Berlin, Takustrasse 6, D-14195 Berlin, Germany

Received August 26, 2006; E-mail: hzi1@usc.edu

Abstract: The crystal structure of the ammonia transport (Amt) protein AmtB at 1.4 Å resolution revealed four ammonia/ammonium (NH_3/NH_4^+) binding sites along the ~20 Å narrow pore. It is an open question whether the bound NH_3/NH_4^+ are neutral (NH_3) or cationic (NH_4^+). On the basis of the AmtB crystal structure, we calculated the pK_a of these four NH_3/NH_4^+ by solving the Poisson−Boltzmann equation. Except for one NH_3/NH_4^+ binding site (Am1) at the entry point of the Amt pore, binding sites are occupied by NH_3 due to lack of energy contributions from solvation, eliminating an existence of charged form NH_4^+ and, inevitably, its potential cation−π interaction. The only two titratable residues in the pore, His168 and His318, are in the neutral charge state. The NH_4^+ charge state at the Am1 site is stabilized by Ser219 functioning as an H-bond acceptor. However, when involving explicit crystal water nearby, the NH_3 charge state is stabilized by the reorientation of Ser219−OH group. This H-bond donor Ser219 significantly decreases the pK_a of NH_3/NH_4^+ at the Am1 site to ~1. The flip/flop H-bond of Ser219 may play a dual role first in binding and subsequently in deprotonating NH_4^+, which is a prerequisite to conduct NH_3 through the Amt pore across the membrane.

Nitrogen is an important nutrient for bacteria, fungi, and plants to synthesize amino acids. In the natural liquid environment, it is solvated as ammonium ion NH_4^+ rather than being available as gaseous ammonia NH_3. At high concentration, cells can maintain a sufficient NH_3/NH_4^+ reservoir by passive permeation through membrane. At low concentration, ammonium transport (Amt) proteins are needed to take up NH_3/NH_4^+ from the environment. Based on the similarity of protein sequence and structure, Amt protein, methylamine permease, and the human rhesus (Rh) factor protein belong to one large protein family. In the mammalians, excretion of NH_4^+ by kidney is of physiological importance, and two Rh proteins RhBG and RhCG are expressed in the mammalian kidney-collecting duct.[1] Thus, understanding the function of Amt protein will give insights also for Rh proteins.

One of the controversial interpretations of experimental results is whether Amt protein transfers uncharged NH_3[2−4] or charged NH_4^+.[5−7] The presence of a 20 Å-long narrow hydrophobic pore revealed in recent crystal structures of AmtB protein from *Escherichia coli* (*E. coli*) at 1.4 Å[4] and 1.8−2.1 Å[8] resolutions strongly suggest that AmtB protein can be considered as channel-conducting gaseous NH_3 rather than an ammonia transporter. In the 1.4 Å AmtB protein structure,[4] four NH_3/NH_4^+ binding sites in the narrow pore were identified (Figure 1, Am1−4).

It has been assumed that the Am1 site close to the periplasmic side is occupied by NH_4^+ rather than NH_3, and NH_4^+ becomes less basic until it reaches the Am2 site.[4] Two reasons have been discussed for a cationic NH_4^+ state at the Am1 site.[9] The Am1 site is relatively polar due to H-bonds with Ser219 and the two crystal waters HOH-573 and HOH-587. It was also proposed that the aromatic residues Phe107 and Trp148 might provide a cation−π interaction to stabilize the charged NH_4^+ [4,10] (Figure 1). In studies of synthetic molecular complexes, NH_4^+ has a larger affinity than NH_3 to tripodal receptor molecules with three pyrazole groups.[11] Due to the similarity of the H-bond pattern of these synthetic molecules with the Am1 site environment, the Am1 site was assumed to be occupied by NH_4^+ rather than NH_3.[9] However, in another Amt protein Amt-1 from *Archaeoglobus fulgidus* (*A. fulgidus*), the corresponding position was

† The Pennsylvania State University.
‡ Free University of Berlin.
§ Current address: 417 SGM Building, Department of Chemistry, University of Southern California, Los Angeles, CA 90089.

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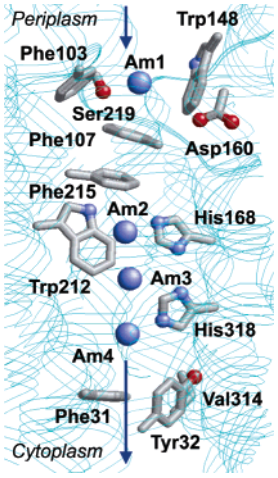

Figure 1. 1. Structural overview of the hydrophobic narrow pore in AmtB based on the crystal structure (PDB code: 1U7G).[4] The four nitrogen atoms of NH_3/NH_4^+ are shown by large spheres. Nitrogen and oxygen atoms of the protein are shown by small spheres.

found not to be occupied by cationic $CH_3NH_3^+$.[12] Hence, the binding affinity of NH_3/NH_4^+ at the Am1 site might depend on specific conditions and may not always be high. In this relation, former computational studies (see Supporting Information in ref 8) indicate that the calculated free energies for NH_3 and NH_4^+ relative to bulk solvent were essentially the same in the region near the Am1 site but were different in regions corresponding to other binding sites Am2−4.

In the present study, we calculated the pK_a of the four NH_3/NH_4^+ binding sites in AmtB protein based on the 1.4 Å structure[4] and report the influence of protein residues on these pK_a values. Although this AmtB structure has three mutated sites, all of them are sufficiently far away from residues generally considered crucial for NH_3/NH_4^+ transfer (Supporting Figure S1).[4] Energetics of electrostatic environment in AmtB protein revealed in the present study will provide further insights of possible NH_3/NH_4^+ binding sites.

Computational Procedures

Atomic Coordinates and Charges. We used the crystal structure of AmtB from *E. coli* with NH_3/NH_4^+ groups at 1.4 Å resolution (PDB code: 1U7G).[4] The atomic coordinates were prepared in the same manner as in previous applications for membrane proton channels of the bacterial photosynthetic reaction center[13,14] or photosystem II.[15]

The position of hydrogen atoms was energetically optimized with CHARMM[16] using the CHARMM22 force field. During this procedure, the positions of all non-hydrogen atoms were fixed, and all titratable groups were kept in their standard charge state, i.e., basic groups were considered protonated and acidic groups ionized. For NH_3/NH_4^+ groups, see the next section. All other atoms whose coordinates were available in the crystal structure were not geometry optimized.

Atomic partial charges of the amino acids were adopted from the all-atom CHARMM22[16] parameter set. For the treatment of atomic charges of titratable residues, see method in the Supporting Information. The charges of protonated acidic oxygen atoms were both increased symmetrically by +0.5 unit charges to account implicitly for the presence of a proton. Similarly, instead of removing a proton in the deprotonated state, the charges of all protons of the basic groups of Arg and Lys were diminished symmetrically by a total unit charge. For residues whose protonation states are not available in the CHARMM22 parameter set, appropriate charges were computed.[17] Charges of NH_3/NH_4^+ are not available in CHARMM22. They were determined from the electronic wave functions by fitting the resulting electrostatic potential in the neighborhood of these molecules by the RESP procedure[18] as we did before with other cofactors.[14] The electronic wave functions were calculated with the DFT module in JAGUAR[19] using the B3LYP functional with 6-31G** basis set (Table S1 in Supporting Information).

Determination of Hydrogen Atom Positions in NH_3/NH_4^+. First, we tentatively started with atomic charges corresponding to the cationic NH_4^+ state for all four NH_3/NH_4^+ binding sites, optimized the hydrogen atom geometry, and calculated the protonation states. On the basis of these resulting tentative protonation states, we performed a second geometry optimization and calculated the final protonation states for the NH_3/NH_4^+ groups. The same results were obtained starting tentatively with atomic charges corresponding to the neutral NH_3 state. To determine the optimized hydrogen atom position for the NH_3/NH_4^+ binding sites where the NH_3 state turned out to be more likely than the NH_4^+ state in the tentative computational results, we first determined the positions of three hydrogen atoms of the NH_3 molecule, fixed their positions as well as those of all the other atoms in protein, and attached a fourth hydrogen atom to the NH_3 group. A fourth hydrogen atom is technically necessary to titrate NH_3/NH_4^+. It carries no charge in the neutral NH_3 state while in the anionic state all four hydrogens symmetrically carry the same positive charge. Geometry optimization and titration of each NH_3/NH_4^+ binding site were performed in the absence of the other three NH_3/NH_4^+ groups at their binding sites. We also did geometry optimization where all binding sites are simultaneously occupied by NH_3/NH_4^+. The resulting protonation patterns are consistent for both cases, i.e., NH_3/NH_4^+ at Am1 is fully protonated while the other sites are fully deprotonated. The present study is based on the former treatment.

Dielectric Volume. As a general and uniform strategy, all crystal waters were removed in our computations[13−15] because of lack of experimental information for hydrogen atom positions (for an exception, see the next section). Cavities resulting after removal of crystal water are uniformly filled with solvent dielectric medium of $\epsilon = 80$. Accordingly, the effect of the removed water molecules was considered implicitly by the high value of the dielectric constant in these cavities. A discussion on the appropriate value of the dielectric constant in proteins for electrostatic energy computations can be found in refs 20−22.

Treatment of Two Crystal Waters at H-Bond Distances from the Am1 Site. Among the four NH_3/NH_4^+ binding sites, only the Am1 site has two crystal waters at H-bond distances, HOH-573 and HOH-587 ($N_{Am1}-O_{water}$ distances = 2.8 and 2.5 Å, respectively, at PDB code 1U7G).[4] Involvement of these two water atoms in geometry optimization does not affect the H-bond pattern between the Am1 site and Ser219 in the case of NH_4^+. For NH_3, HOH-587 affects the H-bond with Ser219 as will be discussed below, while there is no effect from water HOH-573. For consistency of electrostatic energy computations, we considered the crystal water HOH-587 situated in the NH_3 pore only for geometry optimization, and removed it like all other crystal

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water for electrostatic energy computations. Their influence is accounted for by placing a high dielectric medium in the resulting cavities as is done routinely in all our electrostatic energy computations.[13−15,22−24]

Computation of Protonation Pattern and pK_a. The computation of the energetics of the protonation pattern is based on the electrostatic continuum model by solving the linear Poisson−Boltzmann (LPB) equation with the program MEAD from Bashford and Karplus.[25] To sample the ensemble of protonation patterns by a Monte Carlo (MC) method, we used our own program, Karlsberg (Rabenstein, B. (1999) *Karlsberg online manual*, http://agknapp.chemie.fu-berlin.de/karlsberg/). The dielectric constant was set to $\epsilon_P = 4$ inside the protein and $\epsilon_W = 80$ for water. For evaluation of the dielectric constant in the protein, see discussion and Table S2 in the Supporting Information. All computations were performed at 300 K with pH 7.0 and with an ionic strength of 100 mM. The LPB equation was solved using a three-step grid-focusing procedure with grid resolutions of 2.5 Å, 1.0 Å, and 0.3 Å. MC sampling yield probabilities of the deprotonated and protonated state of the considered titratable residues. With the Henderson−Hasselbalch equation, the pK_a can be calculated as the pH where the concentrations of the protonated and deprotonated residue species are equal (Henderson−Hasselbalch pK_a). We used a pK_a of 9.25[26] for the NH_3/NH_4^+ group in aqueous solution as the reference model system.

Error Estimation. The procedures to compute pK_a of titratable residues are equivalent to those of the redox potential for redox-active groups, although in the latter case, the Nernst equation is applied instead of the Henderson−Hasselbalch equation.[27] Therefore, the accuracy of the present pK_a computations is directly comparable to that obtained for recent computations.[13−15] From the analogy, the numerical error of the pK_a computation can be estimated to be about 0.2 pH units. Systematic errors typically relate to specific conformations that may differ from the given crystal structures.

Results and Discussions

NH_3 or NH_4^+. The calculated protonation pattern shows that the Am1 site at the entry point of the narrow pore is a fully protonated NH_4^+ and that the other three sites—Am2, Am3, and Am4—are occupied by fully deprotonated NH_3. The calculated pK_a of the Am1 site (pK_a(Am1)) is 9.1, which is as high as NH_3/NH_4^+ in bulk solvent. In contrast, those of the Am2, Am3, and Am4 sites are significantly lower, namely −2.8, −4.9, and 3.6, respectively (Table 1), in agreement with the proposed mechanism of Khademi et al.[4] that the Am1 is a cationic NH_4^+ until it reaches the Am2 site.

Although the calculated pK_a(Am1) is 9.1, close to 9.25[26] in bulk solvent, it does not imply that the Am1 site is free from protein interactions. For instance, when we eliminate the atomic charges of the AmtB protein but keep the dielectric volume of the atoms (i.e., electrostatic energy computation with a charge free protein), we obtained pK_a(Am1) = 1.8 (Table 1). Thus, uncharged low dielectric of the protein volume significantly decreases the pK_a(Am1) by ∼7 units with respect to aqueous solution (Table 1) mainly due to lack of energy contributions from solvation.[21] In contrast, protein atomic charges upshift the pK_a(Am1) by ∼7 units, compensating for the decrease in the pK_a(Am1) caused by the low dielectric of protein volume (Table 1). Note that such a compensation of the two interactions is very specific to the Am1 site, not to the other sides (see below). Protein backbone dipoles, especially those of trans-membrane helices sometimes have significant impact on the energetics of titratable sites. For instance, in the photosynthetic protein−pigment complex Photosystem I, the protein backbone dipoles down-shift the redox potential of chlorophyll *a* by 150 to 180 mV.[22] However, in AmtB protein, the direct influence of protein backbone to pK_a(NH_3/NH_4^+) is relatively small with respect to that of side chains (Table 1).

Table 1. Calculated pK_a(NH_3/NH_4^+) in the Four Binding Sites[a] and Direct Contribution of Residue Charges to the pK_a(NH_3/NH_4^+)

Calculated pK_a					
	Ser219[b] to Phe215[c]				Ser219[c] to Am1[b]
H-bond pattern	Am1	Am2	Am3	Am4	Am1
pK_a for charged protein[d]	9.1	−2.8	−4.9	3.6	1.2
NH_3 or NH_4^+	NH_4^+	NH_3	NH_3	NH_3	NH_3
pK_a for uncharged protein[e]	1.8	−4.4	−4.4	−3.6	2.0

Contribution of Residues' Charges to the pK_a(NH_3/NH_4^+)					
	atomic charges				
residue	Am1	Am2	Am3	Am4	Am1
whole protein[f]	+7.3	+1.6	−0.5	+7.2	−0.8
Phe31				+0.5	
Tyr32				+0.5	
Phe103	−0.1				−0.1
Phe107	+0.3				+0.3
Trp148	+0.6				+0.4
Asp160	+2.0				+2.3
His168		−2.9	+0.5		
Trp212		+1.3	+0.3		
Phe215		+0.9			
Ser219	+2.3				−6.2
Val314				+0.1	
His318			−2.7	+6.5	
other sidechains	+1.0	+2.9	+2.1	+0.5	+1.2
backbone	+1.2	−0.6	−0.7	−0.9	+1.3

[a] According to ref 4. [b] As an H-bond acceptor. [c] As an H-bond donor. [d] Atomic charges and van der Waals volumes are considered (standard condition of electrostatic energy computations in protein). [e] To investigate the influence of atomic charges on pK_a, atomic charges are set to zero while van der Waals volumes are considered. [f] Direct influence of all protein atomic charges on calculated pK_a.

Asp160 Stabilizes NH_4^+ State at the Am1 Site. A residue that has a significant influence on the Am1 site in the present computation is Asp160 (Figure 1), which up-shifts the pK_a of the Am1 site by 2.0 units (Table 1). Asp160 itself is not accessible to the bulk solvent.[4] Nevertheless, our computations result in a fully deprotonated Asp160, which is stabilized by H-bonds from backbone amide nitrogens of Gly163, Gly164, and Thr165 with $O_{carboxyl}$−$N_{backbone}$ distances of 2.8, 2.8, and 2.7 Å, respectively.

Although the two carboxyl oxygens of the Asp160 side chain are not very close to the Am1 site ($O_{carboxyl}$−N_{Am1} distances of 7.6 and 8.3 Å), it is the only charged residue near the Am1 site (Figure 1). The calculated direct influence of Asp160 on the pK_a(Am1) implies that a loss of the negative charge at Asp160 by mutation to an uncharged residue would decrease the pK_a of the Am1 site by 2 and thus may yield a pK_a(Am1) of ∼7, leading possibly to an increased probability of uncharged NH_3 at this site. However, the occurrence of a neutral NH_3 at the Am1 site may not be so likely, since the Am1 site is still partially exposed to bulk solvent where NH_4^+ is the dominant species (for an exception, see below). Thus, the affinity of the Am1 site to bind NH_3/NH_4^+ may be reduced if Asp160 is replaced by an uncharged residue, probably going along with a decrease in NH_3/NH_4^+ conduction efficiency. In agreement with our

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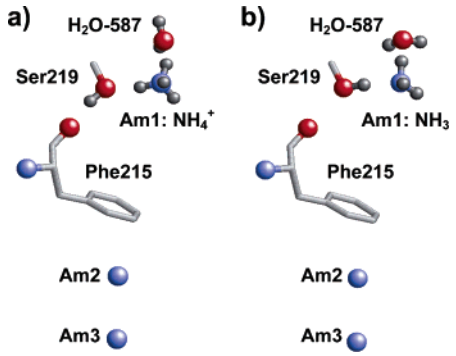

Figure 2. 2. H-bond pattern at the Am1 site: (a) for NH_4^+ and (b) for NH_3. Polar hydrogen atoms involved in H-bonds are displayed explicitly by small-sized spheres. Nitrogen and oxygen atoms are shown by medium-sized spheres.

suggestion, Javelle et al.[28] has demonstrated that mutation of the conserved Asp160 with Ala (D160A) blocks $CH_3NH_2/CH_3NH_3^+$ transport completely.

Previously, it was speculated that Asp160 is a primary binding site for NH_4^+,[28,29] but Khademi et al.[4] did not observe any signal of NH_4^+ binding at Asp160 even at higher concentrations of $(CH_3NH_3)_2SO_4$. The same conclusion was obtained from structural studies of Zheng et al.[8] Our computational results imply that negatively charged Asp160 can provide an appropriate driving force for cationic NH_4^+ binding at the Am1 site, although this residue is relatively distant from the Am1 site (about 8 Å). Mutation from uncharged to charged residues may result in long-range conformational changes of protein structures. If the protein structure of this region in the mutant AmtB remains unchanged, the main factor to fail $CH_3NH_2/CH_3NH_3^+$ transport upon the mutation is likely the electrostatic role of Asp160. The crystal structure of the D160A AmtB is currently unavailable and will be needed to understand this issue.

Ser219 as a Possible H-Bond Acceptor of the Am1 Site. In the crystal structure,[4] the hydroxyl oxygen of Ser219 is at H-bond distance from the Am1 nitrogen atom ($O_{hydroxyl}-N_{Am1}$ distance of 2.8 Å). In the present study, geometry optimization for the NH_4^+ state at the Am1 site with CHARMM[16] results in an H-bond where Ser219 accepts an hydrogen from NH_4^+ at the Am1 site, in agreement with recent quantum chemical studies at the MP2/6-311+G* level.[10] Thus, in the present study, Ser219 has the largest impact on the pK_a(Am1), up-shifting the pK_a(Am1) by 2.3 and stabilizing the cationic NH_4^+ state (Table 1). The hydroxyl hydrogen of Ser219 is oriented to the protein backbone carbonyl oxygen of Phe215 ($O_{hydroxyl}-O_{backbone}$ distance = 2.7 Å) (Figure 2a).

Cation$-\pi$ Interaction and Solvation Energy. NH_4^+ at the Am1 site must become deprotonated before approaching the relatively hydrophobic region of the Am2 site.[4,12] Inspecting the crystal structure, the Am1 site is clearly isolated from the Am2 site by two bulky hydrophobic residues, Phe107 and Phe215 (Figure 1). The B-factor of Phe204 in the crystal structure for *A. fulgidus* Amt-1 (Phe215 in *E. coli* AmtB) is significantly elevated compared with the surrounding residues,[12] implying a higher mobility of this Phe. Guiding a charged NH_4^+ through the Phe107/Phe215 moiety is energetically probably less favorable than an uncharged NH_3.

It is well-known that cation$-\pi$ interactions can be relatively strong both in the gas phase and in aqueous media (reviewed in ref 30). A cation$-\pi$ interaction can only occur if the NH_4^+ charge state is stable. This holds true for studies based on synthetic model complexes much smaller than proteins where NH_4^+ is fully solvated by bulk solvent.[11] In contrast, the contribution of solvation energy to stabilize the charged NH_4^+ is likely too small inside the pore (see also ref 21 for discussion) where the bulky hydrophobic residues Phe107 and Phe215 are located. Charged NH_4^+ is likely to release its proton while entering the pore because of small solvation energy. Majority of proposals for an important role of cation$-\pi$ interactions in the hydrophobic pore of AmtB[4,10] seem to ignore the little existence of charged NH_4^+ there, an essential condition of cation$-\pi$ interactions.[30] Hence, deprotonation of NH_4^+ coupled with entering the hydrophobic pore is energetically more favorable than even utilizing cation$-\pi$ interaction between aromatic rings and a charged NH_4^+. It seems that there is no strong reason to consider the existence of a charged NH_4^+ in the hydrophobic pore at the Am2 site.

Ser219 as a Transient H-Bond Donor at the Am1 Site. We found that geometry optimization for uncharged NH_3 at the Am1 site with CHARMM[16] results in a reorientation of the Ser219 hydroxyl group toward the NH_3 nitrogen at the Am1 site (Figure 2) when crystal waters are considered explicitly in the computation. Involvement of a single-crystal water HOH-587 at 2.5 Å from the Am1 nitrogen atom is sufficient for both reorientation and H-bond formation of Ser219 (Figure 2b). In the absence of HOH-587, such a flip/flop H-bond of Ser219 is not observed upon change of NH_3/NH_4^+ charge state.

In the presence of an H-bond donating Ser219 to NH_3 (Figure 2b), we calculated the pK_a(Am1) to be 1.2 (Table 1), in agreement with the assumption (prior to titration) of a deprotonated NH_3 at the Am1 site. The significant decrease of pK_a(Am1) by 7.9 (with respect to 9.1) (Table 1) originates predominantly from (i) the flip/flop of the Ser hydroxyl hydrogen and (ii) highly packed protein dielectric environment along the hydrophobic pore. The calculated direct influence on the pK_a(Am1) indicates that an H-bond acceptor Ser219 to the Am1 nitrogen atom down-shifts the pK_a(Am1) by 6.2, while an H-bond donor Ser219 up-shifts the pK_a(Am1) by 2.3 (Table 1). Note that the protonation states of the other titratable sites remain essentially unchanged upon H-bond rearrangement of Ser219.

We also calculated pK_a(Am1) in a model system that consists of only Am1 and Ser219, being fully solvated by water (i.e., in the absence of other atoms in the protein). The calculated shift in pK_a(Am1) with the flip/flop of the Ser hydroxyl hydrogen in this system is considerably small, 3.6. This shift, equivalent to ~200 mV, is in a normal range of electrostatic influence of a strong H-bond on the titratable site in protein environment.[24,31] Thus, we emphasize that the unusually large decrease of pK_a(Am1) by 7.9 in AmtB protein is not an artifact but the result of the significantly highly packed protein dielectric environment along the hydrophobic pore that prevents energy contributions from solvation.[21]

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Flip/Flop H-bond of Ser219 for NH_4^+ Binding and NH_4^+ Deprotonation. The present result implies dual roles of Ser219 at the Am1 site. First, as an H-bond acceptor, this residue can facilitate the fixation of NH_4^+ at the Am1 site and can play a role in compensating for the loss in entropy accompanied by transferring NH_4^+ from bulk solvent into the hydrophobic channel. After fixation of NH_4^+ at the Am1 site, Ser219 can act as an H-bond donor and catalyze NH_4^+ deprotonation. This action is coupled with appropriate reorientations of nearby water molecules. The general scheme of this process is comparable to catalytic reactions in enzymes: Fixation of the substrate maximizes the reaction probability by reducing the free energy of the transition state of the reaction. The resulting deprotonated NH_3 can easily diffuse into the hydrophobic pore and reach the Am2 site (Scheme 1).

Scheme 1

$$\text{Ser-OH} + NH_4^+ \text{ (in bulk)} \rightarrow \text{Ser-HO}\bullet\bullet\bullet H^+\text{-}NH_3 \leftrightarrow [\text{Ser-OH}\bullet\bullet\bullet NH_3 + H^+] \rightarrow \text{Ser-OH} + NH_3 \text{ (in pore)} + H^+ \text{ (in bulk)}$$

The meta-stable NH_3 state at the Am1 site may have lower binding affinity than the NH_4^+ state. Interestingly, in the crystal structure of Amt-1 from *A. fulgidus*[12] no electron density of $CH_3NH_3^+$ at the position corresponding to the Am1 site of AmtB was found in spite of the large structural similarity between the two proteins. On the other hand, in former computational studies (see Supporting Information in ref 8), the calculated free energies for NH_3 and NH_4^+ relative to bulk solvent were essentially the same in the region near the Am1 site, implying that binding of both NH_3 and NH_4^+ are energetically possible (Scheme 1). Hence, the NH_3 affinity at the Am1 site depends on specific conditions and might not always be high. The presence or absence of NH_3/NH_4^+ at the Am1 site between *E. coli* AmtB[4] and *A. fulgidus* Amt-1[12] structures may be related to the occurrence of a flip/flop H-bond of Ser219 under different conditions.

Ser219 is highly conserved among Amt proteins (e.g., Amt-1,-2, and -3 proteins). In the human Rh factor proteins that belong to the same large protein family, this position is not occupied by Ser (Leu for RhBG and Ile for RhAG and RhCG), but Ser or Thr residues are always nearby (Thr at 220 for RhBG, Ser at 217 for RhAG, and Ser at 217 and 220 for RhCG). Since the crystal structure for Rh proteins is not available yet (for modeling studies of Rh proteins, see ref 32), the exact location of these Ser or Thr in protein structures are unclear. However, considering significant protein sequence identity shared among Amt and human Rh proteins, positions 217 and 220 in human Rh proteins are probably at the vicinity of the hydrophobic pore entrance. Thus, we propose that an equivalent function of Ser (Thr) commonly serve NH_4^+ binding and deprotonation among these families. Future mutational studies of these Ser and Thr, especially Ser219 in AmtB protein, could provide support for the proposed reaction mechanism.

Physiological Importance of a Flip/Flop H-Bond of Ser in Other Biological Systems. The importance of a flip/flop H-bond of Ser (or Thr) hydroxyl groups that can tune the energetics of cofactors in proteins has been discussed in other systems. In bacterial photosynthetic reaction centers, Ser-L223 is at an H-bond distance from the electron acceptor secondary quinone Q_B. In response to charge states nearby, Ser-L223 forms an H-bond either with the carboxylic oxygen atom Asp-L213 or the carbonyl oxygen atom of Q_B.[23,33,34] Since the formation of the H-bond between Ser223 and Q_B stabilizes the anionic form Q_B^- and up-shifts the redox potential of Q_B by ~100 mV,[23] it was suggested that this flip/flop H-bond of Ser-L223 can play a significant role in gating the electron-transfer event between primary and secondary quinone in bacterial photosynthetic reaction centers.[23,35] In the photosynthetic pigment−protein complex among cyanobacteria and plants, photosystem II, a flip/flop H-bond of D2-Thr217 can change the redox potential of Q_A,[24] which may play a photoprotective role by avoiding the harmful triplet generating charge-recombination pathway.[36−38]

His168 and His318 in the Hydrophobic Pore. His168 and His318 are highly conserved and the only titratable residues located at the center of the hydrophobic pore near the Am2, Am3, and Am4 sites (Figure 1). On the basis of the remarkable conservations of the two His residues, it has been speculated that the two His residues may facilitate deprotonation of NH_4^+ to NH_3.[8] The calculated direct influence indicates that His169 down-shifts the pK_a(Am2) by 2.9 units (Table 1), implying that His169 could facilitate deprotonation of NH_4^+ if the protonated cation was able to reach this histidine. However, in reality, such deprotonation of NH_4^+ might not occur because NH_4^+ is deprotonated at the Am1 site (Scheme 1) and only deprotonated NH_3 can reach this histidine.

In the present study, both His168 and His318 are permanently in the charge-neutral deprotonated state as well as NH_3 being uncharged at the Am2, Am3, and Am4 sites. The Nδ site of His168 and Nϵ site of His318 are permanently deprotonated, in agreement with the proposal by Khademi et al.[4] Furthermore, even if we constrain the NH_3/NH_4^+ state at the Am2, Am3, or Am4 sites to be the cationic NH_4^+, we observed neither a significant change of protonation states for titratable residues, including the two histidines, nor changes of the Nδ/Nϵ tautomer states for His168 and His318 with respect to an equilibrium state where these binding sites are occupied by NH_3. Hence, protonation events are unlikely to occur near these histidines in the pore due to absence of solvation.[21]

Conclusion

The calculated pK_a for the NH_3/NH_4^+ binding sites indicate that a charged NH_4^+ is at the Am1 site and uncharged NH_3 is at the other sites (Am2, Am3, Am4). The protein dielectric volume rather than the protein atomic charges are the dominant factors determining whether NH_3 or NH_4^+ is energetically favorable at each binding site. The anionic state of Asp160 stabilizes the cationic NH_4^+ state at the Am1 site, suggesting its significant contribution to NH_4^+ stabilization and fixation. Involvement of a crystal water in an H-bond distance from the

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Am1 site results in donation of an H-bond from Ser219 to the nitrogen atom of uncharged NH_3 at the Am1 site, decreasing the pK_a(Am1). The present results suggest that Ser219 plays a dual role in governing NH_3/NH_4^+ conduction through the NH_3 pore. (i) With support from the anionic Asp160, it firmly attaches NH_4^+ at the Am1 site acting as an H-bond acceptor and thus compensates for loss of entropy from immobilization of NH_4^+. (ii) It deprotonates NH_4^+ by flipping from an H-bond acceptor to an H-bond donor mode. A deprotonation of NH_4^+ to NH_3 synchronized with entering the hydrophobic pore should be energetically more favorable than maintaining the protonated form of NH_3 (i.e., NH_4^+) due to limited availability of solvation in the pore.

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Supporting Information Available: Discussion about relations between pK_a and protonation patterns, and atomic charges for NH_3/NH_4^+. This material is available free of charge via the Internet at http://pubs.acs.org.

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