Hydronium Ions

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The Existence of an Isolated Hydronium Ion in the Interior of Proteins

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Abstract: *Neutron diffraction analysis studies reported an isolated hydronium ion (H_3O^+) in the interior of D-xylose isomerase (XI) and phycocyanobilin-ferredoxin oxidoreductase (PcyA). H_3O^+ forms hydrogen bonds (H-bonds) with two histidine side-chains and a backbone carbonyl group in PcyA, whereas H_3O^+ forms H-bonds with three acidic residues in XI. Using a quantum mechanical/molecular mechanical (QM/MM) approach, we analyzed stabilization of H_3O^+ by the protein environment. QM/MM calculations indicated that H_3O^+ was unstable in the PcyA crystal structure, releasing a proton to an H-bond partner His88, producing H_2O and protonated His88. On the other hand, H_3O^+ was stable in the XI crystal structure. H-bond partners of isolated H_3O^+ would be practically limited to acidic residues such as aspartic and glutamic acids in the protein environment.*

Water molecules can serve as the proton donor and acceptor in the hydrogen bond (H-bond) network of the protein interior, forming a proton transfer pathway with titratable residues. In particular, when water molecules are strongly H-bonded, the activation energy for proton transfer is the lowest, without involving formation of an isolated hydronium ion, H_3O^+.[1] On the other hand, H_3O^+ was proposed to be present in H, K-ATPase[2] or at the end of the proton transfer pathway in bacteriorhodopsin[3] (but see also Ref. [4]). Neutron diffraction analysis of rubredoxin suggested the presence of H_3O^+ on the protein surface.[5] In all these examples, it is assumed that H_3O^+ is stabilized by the donation of OH groups to the acceptor water molecules (for example, $[H_2O\cdots H\cdots OH_2]^+$). In contrast, "isolated H_3O^+", which exists in the absence of other water molecules, was not identified in protein crystal structures until neutron diffraction analysis of metal-removed D-xylose isomerase (XI)[6] and the more recent neutron diffraction analysis of phycocyanobilin-ferredoxin oxidoreductase (PcyA)[7] were reported. It has been proposed that in the PcyA neutron structure, H_3O^+ donates H-bonds to Nδ of His74, Nε of His88, and the backbone carbonyl O of Leu243 (Figure 1a).[7] The H-bond network of the H_3O^+-binding moiety is also proposed to be involved in the proton transfer pathway, which would be necessary for the endovinyl reduction of biliverdin IXα.[8] In the XI neutron structure, H_3O^+ was proposed to donate H-bonds to Glu181, Glu217, and Asp245, which was originally the metal-binding moiety of the enzyme[6] (Figure 1b). The existence of isolated H_3O^+ is possible only when pK_a of H_3O^+/H_2O [$pK_a(H_3O^+)$] is higher than that of all of the H-bond acceptor groups. However, in water, $pK_a(H_3O^+)$ is −1.7, which is significantly lower than that for Asp (4.0), Glu (4.4), Nδ of His (6.6), and Nε of His (7.0) in water.

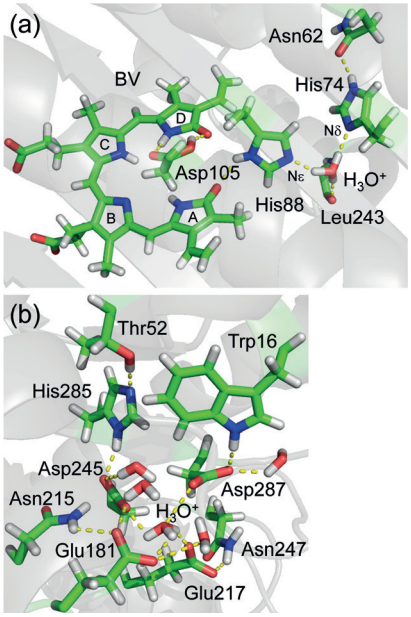

Figure 1. H_3O^+-binding sites proposed by neutron diffraction analysis in a) PcyA (PDB code: 4QCD) and b) XI (PDB code: 3KCJ). Yellow dotted lines indicate H-bond interactions.

The pK_a of H-bond donor and acceptor moieties in H-bonds can be analyzed from the potential energy profiles of the H-bonds (Supporting Information, Figure S1).[9] In H-bonds, a proton is more likely to populate the moiety with the higher pK_a value between the two moieties (Supporting Information, Figure S2).[9c] The energy difference between the H-bond donor and acceptor moieties corresponds to the pK_a difference (Supporting Information, Figure S3). This feature also holds true for H-bonds in protein environments,[9b,c,10] which are typically analyzed at the density functional theory (DFT) level. Calculations performed at the DFT level are likely to stray away from correct

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description of geometry towards better description of energy.[11] Therefore, H-bonds should be evaluated based on not only the distances but also the potential-energy profiles, as suggested by Schutz and Warshel.[9b] In particular, low-barrier H-bonds (LBHB), which also exist in $[H_2O\cdots H\cdots OH_2]^+$, can be unambiguously defined by the potential-energy profile at the DFT level (Supporting Information, Figure S4), because identical pK_a values of the donor and acceptor moieties is the requirement for LBHB formation (that is, asymmetric single minimum H-bonds are not LBHB, as suggested by Schutz and Warshel[9b]). Further description of the H-bonds may be obtained with the solution of the nuclear Schrödinger equation.[12]

Herein, to understand how the protein environment can stabilize an isolated H_3O^+, we analyzed the potential energy profiles of H-bonds in the proposed H_3O^+ binding moieties by adopting a quantum mechanical/molecular mechanical (QM/MM) approach based on the neutron structures of PcyA and XI.

The stability of H_3O^+ in PcyA was investigated. The neutron structure of PcyA has two conformers: conformer I, corresponding to the case with protonated biliverdin and ionized Asp105; and conformer II, corresponding to deprotonated biliverdin and protonated Asp105.[7] QM/MM calculations indicated that H_3O^+ at the His74, His88, and Leu243 moiety was unstable, and that in both conformers I (Figure 2; Supporting Information, Table S1) and II (Supporting Information, Table S2), it released a proton to Nε of His88 to form H_2O and doubly protonated His88; the resulting geometries were practically the same in the two conformers. Below, we focus on conformer I.

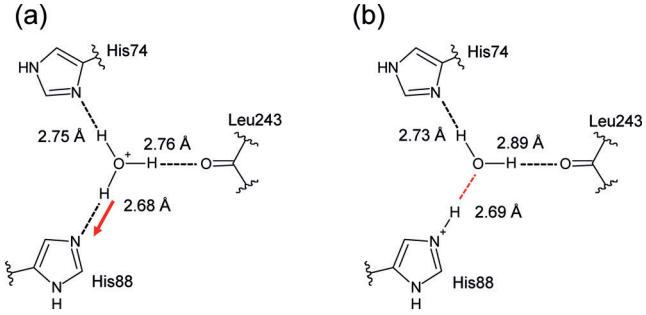

Figure 2. H_3O^+ binding sites proposed by neutron diffraction analysis of PcyA a) in the neutron structure and b) the QM/MM-optimized geometry. The red dotted line indicates the newly formed H-bond in the QM/MM-optimized geometry.

Notably, the H-bond lengths O_{water}–$N\varepsilon_{His88}$ and O_{water}–$N\delta_{His74}$ in the original neutron structure (2.7 and 2.8 Å[7]) were reproduced even in the QM/MM-optimized geometry, where H_3O^+ was absent but H_2O and doubly protonated His88 were present (2.7 and 2.7 Å, respectively; Supporting Information, Table S1). Thus, the geometry of the heavy atom position in the neutron structure can be explained without assuming the presence of H_3O^+. Although the present study suggests that H_3O^+ is absent in the PcyA neutron structure, one H^+ shows high probability of existence at the $N\varepsilon_{His88}$ moiety. When assuming H_2O, deprotonated $N\varepsilon_{His88}$, and deprotonated $N\delta_{His74}$, the resulting root-mean-square deviations (RMSD) of the QM/MM-optimized geometry from the neutron structure were significantly large (Supporting Information, Table S1).

The potential energy profile of H-bonds also indicated that in the $O_{H_3O^+}$-O_{Leu243} H-bond, the proton is located at the H_3O^+ moiety, that is, $pK_a(O=C_{Leu243}) < pK_a(H_3O^+)$ (Figure 3). On the other hand, in the $O_{H_3O^+}$–N_{His74} and $O_{H_3O^+}$–N_{His88} H-bonds, the protons are located at the histidine moieties (Figure 3). The energy difference between the H_3O^+ and His88 moieties is larger than that between the H_3O^+ and His74 moieties, which suggests $pK_a(O=C_{Leu243}) < pK_a(H_3O^+) < pK_a(N\delta_{His74}) < pK_a(N\varepsilon_{His88})$ in the PcyA protein environment (Figure 3). In water, $pK_a(H_3O^+)$ is −1.7 and $pK_a(N\varepsilon_{His})$ is about 7. The proposed H_3O^+ binding site in PcyA does not have acidic residues; hence, $pK_a(H_3O^+)$ cannot be increased to overcome the original pK_a difference of over 8 pK_a units (see similar discussions in Ref. [4]). His88 can accept an H-bond from H_3O^+ but cannot decrease $pK_a(H_3O^+)$ to $pK_a(N\varepsilon_{His88})$ because of the absence of a negative charge. The nearest acidic residue Glu76 is about 7 Å away from the proposed H_3O^+ binding site.

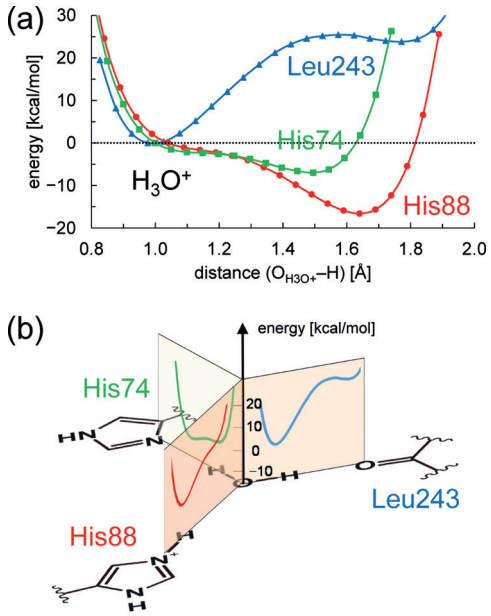

Figure 3. Energy profiles along the H-bonds in the H_3O^+-binding moiety in PcyA. a) Detailed energy profiles. b) Energy profiles along the O···H···O bond axes.

The reasons for the higher $pK_a(N\varepsilon_{His88})$ as compared to $pK_a(N\delta_{His74})$ are the presence of 1) Asp105 near His88 (3.7–5.5 Å[7]), which stabilizes the doubly protonated His88; and 2) Lys72 near His74 (5.1 Å[7]), which destabilizes the doubly protonated His74 (Table 1).

For the presence of stable H_3O^+ at the PcyA binding moiety, $pK_a(N\delta_{His74})$ and $pK_a(N\varepsilon_{His88})$ must be lower than $pK_a(H_3O^+)$, that is, Nδ of His74 and Nε of His88 must be deprotonated. The absence of protonation at Nδ of His74 in

Table 1: Residues that shift $pK_a(N\varepsilon_{His88})$ by more than 2 pK_a units in PcyA.[a]

	$\Delta pK_a(N\varepsilon_{His88})$	$\Delta pK_a(N\delta_{His74})$
Asp105	4.6	1.9
Asp245	2.1	2.5
Lys72	−3.2	−6.0

[a] For comparison, influences of the same residues on $pK_a(N\delta_{His74})$ are also shown. See the Supporting Information for experimental procedures.

the H-bond potential-energy profile (Figure 3) is consistent with the interpretation based on the neutron diffraction analysis reported by Unno et al.[7] On the other hand, the protonated Nε of His88 in the H-bond potential energy profile (Figure 3) is inconsistent with the interpretation based on neutron diffraction analysis. Since the neutron diffraction analysis was carried out in crystals, unstable H_3O^+ might have been be trapped in them. If this is the case, the crystallographers are urged to make efforts to confirm their "interpretation" by chemical data (for example, pK_a, chemical shift). In support of the presence of H_3O^+, Unno et al. stated[7] that "in nuclear magnetic resonance (NMR) studies by Kohler et al.,[13] His88 was reported to be singly protonated in native PcyA". However, according to the original report by Kohler et al.,[13] it was the D105N mutant that had singly protonated His88, and not the native PcyA.[13] His88 was doubly protonated in biliverdin-free native PcyA; the protonation state of His88 could not be determined for biliverdin-bound native PcyA.[13] Even in biliverdin-bound D105N mutant, where His88 was confirmed to be singly protonated at Nε,[13] the experimentally measured NMR chemical shift of 7.86 ppm for the $^1H\varepsilon$ of His88 was far from those observed for LBHB (typically 17–22 ppm;[14] Supporting Information, Figure S4). It is likely that the NMR results[13] do not directly support the interpretation by Unno et al.[7] for the presence of H_3O^+ and deprotonated $N\varepsilon_{His88}$.

The stability of H_3O^+ in XI was then investigated. QM/MM calculations reproduced the presence of H_3O^+, observed as D_3O^+ in the XI neutron structure (Supporting Information, Table S3). The neutron structure could be interpreted to indicate that Glu181, Glu217, and Asp245 are the H-bond acceptors of H_3O^+.[6] On the other hand, the RMSD of the QM/MM-optimized geometry from the neutron structure was the lowest, at 0.27 Å, when Glu217, Asp245, and Asp287 were the H-bond acceptors of H_3O^+ (Supporting Information, Table S3). All the other H-bond patterns resulted in RMSD of 0.35–0.39 Å, which are even higher than the value (0.33 Å) obtained assuming the presence of NH_4^+ (Supporting Information, Table S4). Below, we focus on this QM/MM-optimized structure, where Glu217, Asp245, and Asp287 are the H-bond acceptors of H_3O^+.

In contrast to the neutron structure of PcyA, the neutron structure of XI shows two remarkably short H-bonds, $O_{H_3O^+}-O_{Asp245} = 2.3$ Å and $O_{H_3O^+}-O_{Glu217} = 2.4$ Å.[6] Intriguingly, QM/MM calculations reproduced this result: $O_{H_3O^+}-O_{Asp245} = 2.5$ Å and $O_{H_3O^+}-O_{Glu217} = 2.5$ Å (Figure 4; Supporting Information, Table S3). In sharp contrast to PcyA, the potential energy profile of the H-bonds indicates that the energy

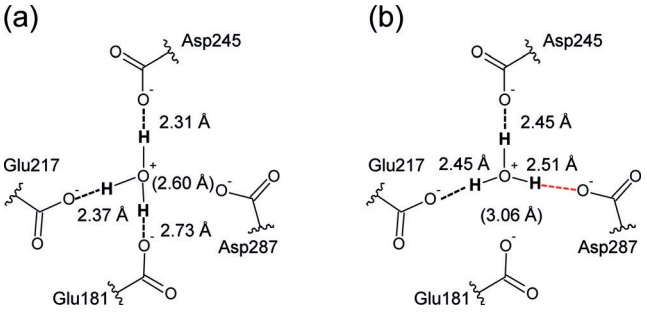

Figure 4. H_3O^+-binding sites proposed by neutron diffraction analysis of XI in a) the neutron structure and b) the QM/MM-optimized geometry with the lowest RMSD. The red dotted line indicates the H-bond that differs between the neutron structure and the QM/MM-optimized geometry.

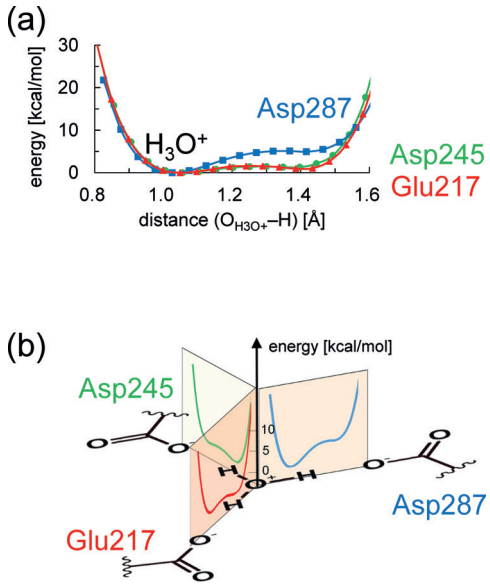

Figure 5. Energy profiles along the H-bonds in the H_3O^+ binding moiety in XI. a) Detailed energy profiles. b) Energy profiles along the O···H···O bond axes.

minimum is localized at the H_3O^+ moiety in all the three H-bonds with an acidic residue (Figure 5), confirming that H_3O^+ exists in the protein interior of XI. The energy difference between the H_3O^+ and proton acceptor moieties suggests that $pK_a(Asp287) < pK_a(Glu217) \approx pK_a(Asp245) \leq pK_a(H_3O^+)$ (Figure 5). In water, $pK_a(Asp, Glu) \approx 4$ is lower than $pK_a(N\varepsilon_{His}) \approx 7$ but still higher than $pK_a(H_3O^+) = -1.7$. In XI, four acidic residues are present at the H_3O^+-binding moiety. These four acidic residues can stabilize the protonated state of H_3O^+ and cause a significant increase in $pK_a(H_3O^+)$, leading to $pK_a(H_3O^+) \geq pK_a(Asp, Glu)$ in XI.

Based on the analysis of the two independent neutron structures, the present study helps in understanding how the two protein environments of the proposed H_3O^+ binding moieties are markedly different.

In XI, the potential energy surface of the H_3O^+ binding moiety, shaped as a symmetric funnel (Figure 6), would be a prerequisite for the existence of isolated H_3O^+. Isolated H_3O^+ is stable in the protein interior only when the energy

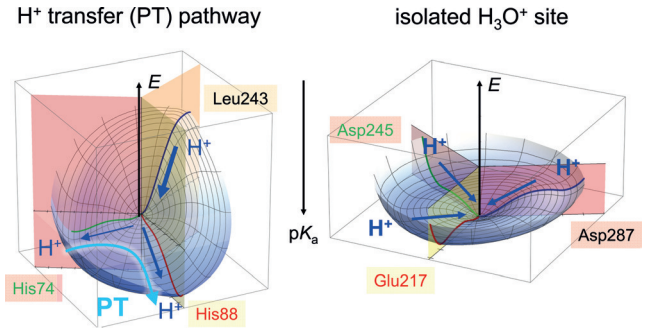

Figure 6. Difference in the energy profiles between proton transfer pathway (PcyA; left) and isolated H_3O^+ binding site (XI; right). Blue arrows indicate migration of H^+ in H-bonds.

minimum of H^+ is localized in the H_3O^+ moiety, that is, pK_a of H_3O^+ must be equal to or larger than pK_a of all the three H-bond acceptors (Figure 6). These H-bond partners would be practically limited to acidic residues such as aspartic acid and glutamic acid in the protein environment.

The potential energy profile of the isolated H_3O^+-binding moiety in XI is in sharp contrast to that of PcyA (Figure 6). In PcyA, deprotonated histidine may accept a H-bond from H_3O^+ but cannot decrease $pK_a(H_3O^+)$ because of the absence of negative charge, thus allowing formation of H_2O and protonated histidine. The backbone carbonyl O at Leu243 is non-titratable; thus, the proton needs to be delocalized over the other two titratable groups His74 and His88 in PcyA, facilitating proton transfer between the two residues. This is consistent with a common view that His74 and the water molecule form a proton transfer pathway (proton shuttle) to His88 in PcyA.[8b,c] Isolated H_3O^+ is unlikely to exist unless the protonated carbonyl of Leu243 is stable in PcyA. Notably, backbone carbonyl groups also exist as H-bond acceptors for water molecules in the proton-conducting water chain of photosystem II,[10b,15] where formation of H_3O^+ is inhibited for efficient proton transfer.[1a]

The proton stabilized in the form of H_3O^+ (for example, XI[6]) may not be readily available as a transferable H^+, that is, catalytically important H^+. Isolated H_3O^+ binding sites may be suitable metal-binding sites, as is originally the case with XI;[6] isolated H_3O^+ plays a role in binding the negatively charged residues and stabilize the protein structure prior to metal binding.

These results may also provide a key to understanding the requirement for the protein environment of efficient proton transfer pathways, for example, photosystem II and bacteriorhodopsin.

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Conflict of interest

The authors declare no conflict of interest.

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[1] a) A. A. Stuchebrukhov, *Phys. Rev. E* **2009**, *79*, 031927; b) V. L. Davidson, *Nat. Chem.* **2011**, *3*, 662–663.
[2] K. Munson, R. Garcia, G. Sachs, *Biochemistry* **2005**, *44*, 5267–5284.
[3] F. Garczarek, K. Gerwert, *Nature* **2006**, *439*, 109–112.
[4] P. Goyal, N. Ghosh, P. Phatak, M. Clemens, M. Gaus, M. Elstner, Q. Cui, *J. Am. Chem. Soc.* **2011**, *133*, 14981–14997.
[5] M. G. Cuypers, S. A. Mason, M. P. Blakeley, E. P. Mitchell, M. Haertlein, V. T. Forsyth, *Angew. Chem. Int. Ed.* **2013**, *52*, 1022–1025; *Angew. Chem.* **2013**, *125*, 1056–1059.
[6] A. Y. Kovalevsky, B. L. Hanson, S. A. Mason, T. Yoshida, S. Z. Fisher, M. Mustyakimov, V. T. Forsyth, M. P. Blakeley, D. A. Keen, P. Langan, *Angew. Chem. Int. Ed.* **2011**, *50*, 7520–7523; *Angew. Chem.* **2011**, *123*, 7662–7665.
[7] M. Unno, K. Ishikawa-Suto, K. Kusaka, T. Tamada, Y. Hagiwara, M. Sugishima, K. Wada, T. Yamada, K. Tomoyori, T. Hosoya, I. Tanaka, N. Niimura, R. Kuroki, K. Inaka, M. Ishihara, K. Fukuyama, *J. Am. Chem. Soc.* **2015**, *137*, 5452–5460.
[8] a) S.-L. Tu, W. Sughrue, R. D. Britt, J. C. Lagarias, *J. Biol. Chem.* **2006**, *281*, 3127–3136; b) S. L. Tu, N. C. Rockwell, J. C. Lagarias, A. J. Fisher, *Biochemistry* **2007**, *46*, 1484–1494; c) B. V. Kabasakal, D. D. Gae, J. Li, J. C. Lagarias, P. Koehl, A. J. Fisher, *Arch. Biochem. Biophys.* **2013**, *537*, 233–242.
[9] a) C. L. Perrin, J. B. Nielson, *Annu. Rev. Phys. Chem.* **1997**, *48*, 511–544; b) C. N. Schutz, A. Warshel, *Proteins Struct. Funct. Bioinf.* **2004**, *55*, 711–723; c) H. Ishikita, K. Saito, *J. R. Soc. Interface* **2014**, *11*, 20130518.
[10] a) K. Saito, J.-R. Shen, T. Ishida, H. Ishikita, *Biochemistry* **2011**, *50*, 9836–9844; b) K. Saito, A. W. Rutherford, H. Ishikita, *Nat. Commun.* **2015**, *6*, 8488.
[11] M. G. Medvedev, I. S. Bushmarinov, J. Sun, J. P. Perdew, K. A. Lyssenko, *Science* **2017**, *355*, 49–52.
[12] M. Nadal-Ferret, R. Gelabert, M. Moreno, J. M. Lluch, *J. Am. Chem. Soc.* **2014**, *136*, 3542–3552.
[13] A. C. Kohler, D. D. Gae, M. A. Richley, S. Stoll, A. Gunn, S. Lim, S. S. Martin, T. I. Doukov, R. D. Britt, J. B. Ames, J. C. Lagarias, A. J. Fisher, *Biochemistry* **2010**, *49*, 6206–6218.
[14] F. Hibbert, J. Emsley, *Adv. Phys. Org. Chem.* **1990**, *26*, 255–379.
[15] T. Takaoka, N. Sakashita, K. Saito, H. Ishikita, *J. Phys. Chem. Lett.* **2016**, *7*, 1925–1932.

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