Neutron Crystallography

International Edition: DOI: 10.1002/anie.201509989
German Edition: DOI: 10.1002/ange.201509989

Long-Range Electrostatics-Induced Two-Proton Transfer Captured by Neutron Crystallography in an Enzyme Catalytic Site

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Abstract: *Neutron crystallography was used to directly locate two protons before and after a pH-induced two-proton transfer between catalytic aspartic acid residues and the hydroxy group of the bound clinical drug darunavir, located in the catalytic site of enzyme HIV-1 protease. The two-proton transfer is triggered by electrostatic effects arising from protonation state changes of surface residues far from the active site. The mechanism and pH effect are supported by quantum mechanics/molecular mechanics (QM/MM) calculations. The low-pH proton configuration in the catalytic site is deemed critical for the catalytic action of this enzyme and may apply more generally to other aspartic proteases. Neutrons therefore represent a superb probe to obtain structural details for proton transfer reactions in biological systems at a truly atomic level.*

Proton transfer is integral to many biochemical processes. However, direct observation and structural characterization of biological proton transfer has not been possible thus far. Proton transfer in aqueous solution is a fundamental chemical reaction that is at the heart of ion channel function and most enzymatic reactions.[1–5] Protons are often relayed between protein residues, water molecules, and substrates. The relay mechanism, or proton "hopping", first proposed by Grotthuss[6,7] to explain anomalously high proton mobility in water, is a generally accepted mechanism for sequential proton transfer in an aqueous medium.

Direct observation of proton transfer in chemical and biological systems is challenging because reaction products may be unstable, or the transferred proton may be exported to the bulk solvent. Hence, ultrafast pump-probe spectroscopic methods, such as femtosecond infrared spectroscopy, have been used to follow the transfer of an acidic hydroxy (OH) proton from a photolabile acid to acetic acid and its derivatives, and even to human serum albumin.[8–11] Similarly, excited-state proton transfer has been studied in green fluorescent protein (GFP), mapping the proton hopping pathway from the fluorophore OH to a glutamate side chain through a water molecule and a serine residue.[12–14] However, in these examples the proton is transferred back to the OH group once the photo-acids return to the ground state and regain their initial acidities.

Unequivocal description of proton transfer mechanisms requires crystallographic experiments on both the reactant and product states. X-rays are scattered by electrons, thus X-ray scattering power increases with atomic number. The smallest atom, hydrogen (H) with only one electron, is therefore difficult to visualize with X-ray crystallography. Accordingly, the most biologically interesting hydrogen atoms are generally not observed in electron density maps,[15] even at sub-angstrom resolution, as exemplified by GFP.[16] Moreover, highly polarized hydrogen atoms with little electron density, and electron-bare protons (H^+), are fundamentally invisible to X-rays. Consequently, no X-ray structural studies have directly observed hydrogen atoms in biomacromolecules before and after occurrence of a proton transfer reaction. In contrast to X-rays, neutrons are scattered by atomic nuclei. The coherent neutron scattering lengths of hydrogen and deuterium (D) are similar in magnitude to those of other atoms found in biomacromolecules. Thus, locating hydrogen and deuterium atoms and ions in macromolecular neutron structures is straightforward, even at resolutions as low as 2.0–2.5 Å.

Macromolecular neutron crystallography (MNC) has been instrumental in answering long-standing questions about enzyme mechanisms[17–19] and ligand binding.[20,21] For example, neutron structures have revealed hydrated protons (or hydronium, H_3O^+), which exist only transiently in bulk water, bound within proteins,[22–24] and have provided indisputable evidence of low-barrier hydrogen bonds in enzyme active sites.[22,25] MNC is hence the method of choice for

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201509989.

unequivocal determination of the positions of hydrogen and deuterium atoms in biomacromolecules.[26] Herein, we demonstrate for the first time that MNC can be used to directly determine hydrogen atom positions in the reactant and product states of a proton transfer reaction; that is, before and after proton transfer. We provide compelling evidence for a two-proton transfer in the confined environment of an enzyme active site within a crystal. Moreover, the proton transfer was found to be triggered by long-range electrostatic effects of surface residues undergoing protonation changes in response to pH.

HIV-1 protease, an aspartic protease, is a key drug target for HIV/AIDS therapy. Understanding its structure and function at the atomic level, including the location and movement of hydrogen atoms, is vital for understanding drug resistance and guiding rational drug design.[27,28] Aspartic proteases catalyze the hydrolysis of peptide bonds by using two closely co-located aspartic acid (Asp) residues (Figure 1).

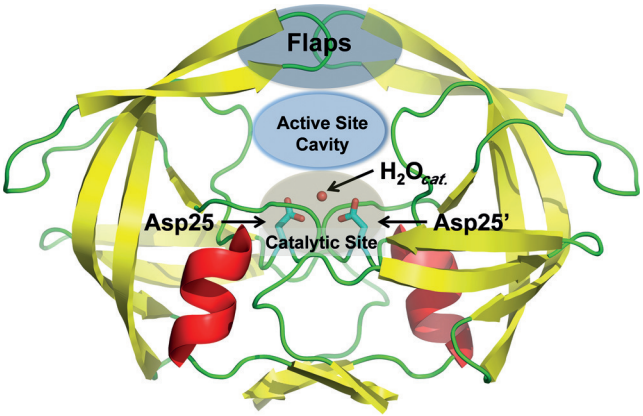

Figure 1. Substrate-free HIV-1 protease showing catalytic Asp25 and Asp25′ residues, and the lytic water molecule. Figure generated from PDB entry 2PC0.

The conventional mechanism for aspartic proteases involves general acid/base catalysis. One protonated Asp carboxylic side-chain (COOH) acts as an acid, donating a proton to the carbonyl oxygen of the scissile peptide bond, while the other deprotonated Asp carboxylate (COO^-) plays the role of a base, and abstracts a proton from the catalytic H_2O.[29] Thus, in the substrate-free form, the catalytic Asp dyad is monoprotonated and bound to an H_2O molecule.[30,31] Quantum chemical calculations place the proton symmetrically between the inner Oδ1 oxygen atoms of the catalytic Asp residues in a low-barrier hydrogen bond, with the catalytic H_2O symmetrically hydrogen-bonded to the two outer Oδ2 oxygen atoms (Figure 2).[32,33] The symmetric hydrogen bonds must be broken so that protease activity can occur, and the proton transferred from the inner to the outer oxygen atom of the Asp. The manner in which the proton shifts from the inner Oδ1 oxygen atoms to the outer Oδ2 of the catalytic Asp residue is a matter of debate. It has been proposed that this process is driven either by substrate binding or by the conformational dynamics of the flap region, a flexible hairpin structure that covers the active site (Figure 1).[31,32]

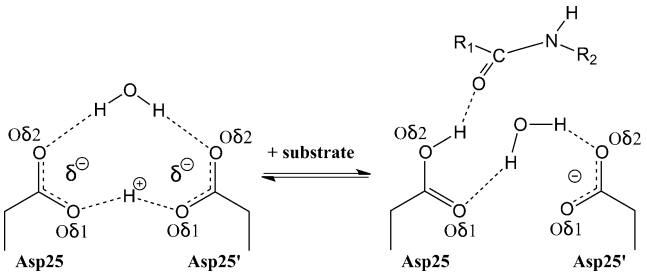

Figure 2. Proposed hydrogen bonding in the catalytic site of substrate-free HIV-1 protease and its rearrangement upon substrate binding.

To directly visualize hydrogen atoms (observed as D) in the catalytic site of an aspartic protease before and after proton transfer, we determined room temperature neutron structures of perdeuterated HIV-1 protease in complex with the clinical drug darunavir at pH 6.0 and 4.3 to resolutions of 2.0 and 2.3 Å, respectively. The omit difference F_O-F_C neutron scattering length density map for the catalytic site in the complex obtained at pH 6.0 indicates that D1 of the catalytic dyad is D^+, located equidistantly between the carboxylate of Asp25′ and the OH of darunavir, with all D–O distances being 1.5–1.6 Å (Figure 3; Supporting Information, Figures S1 and S2). The position of D^+ implies that it

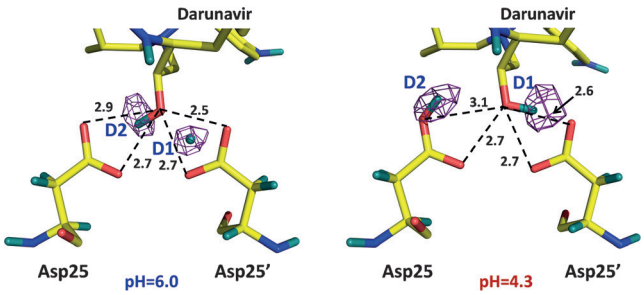

Figure 3. Omit F_O-F_C difference (purple mesh) neutron scattering length density maps (contoured at 3.0 σ) for the HIV-1 protease catalytic site at pH 6.0 (left) and pH 4.3 (right). A two-proton transfer has occurred; that is, D1 on Asp25′ has moved to the OH of darunavir and D2 has been transferred to Asp25. Deuterium atoms (teal), darunavir hydrogen atoms omitted for clarity. Distances are in Å.

is shared by the three oxygen atoms, suggesting possible formation of a low-barrier hydrogen bond. The OH group of the drug faces Asp25, forming a bifurcated hydrogen bond with both carboxylic oxygen atoms (D···O distances of 2.0 Å). The locations of the deuterium atoms in this structure differ from those observed in our recent neutron structure of wild-type HIV-1 protease in complex with amprenavir at pH 6.0, in which the carboxylic deuterium is bound to the inner Oδ1 of Asp25, and the hydroxy deuterium of amprenavir is oriented toward the outer Oδ2 of Asp25′ (Supporting Information, Figure S2)[34] with both deuterium atoms involved in short, but conventional, strong hydrogen bonds, with D···O distances of 1.5 and 1.7 Å.

In the neutron structure at pH 4.3, the four surface residues Asp30, Asp30′, Glu34, and Glu34′, each gained

a proton (Supporting Information, Figure S3), but no other changes in protonation states were observed. Furthermore, D1 and D2 in the active site occupy different positions from those in the pH 6.0 structure (Figure 3). In the pH 4.3 structure, Asp25 is protonated on the outer Oδ2 oxygen, whereas the OH group of darunavir hydrogen bonds with Asp25′. Therefore, a net two-proton transfer has occurred between the catalytic Asp residues and the OH group of the drug. This transfer was triggered by changes in the electrostatic properties of the protein resulting from the protonation of the four surface residues. The electrostatic changes occur relatively far away (11–14 Å) from the catalytic site (Supporting Information, Figure S4). The catalytic site is not accessible to water when an inhibitor is bound, therefore we can deduce the proton transfer pathway. D1 is transferred to the darunavir oxygen, while D2 shifts to Asp25. At low pH, D1 forms a short, strong hydrogen bond with the outer Oδ2 of Asp25′ (D···O distance = 1.6 Å), but D2 only engages in a weak interaction with the darunavir hydroxy oxygen (D···O distance = 2.5 Å), similar to the previously determined pH 4.6 structure of HIV-1 protease complexed with an inhibitor.[35] Importantly, although deuterium atoms are found in disparate arrangements, the relative positions of the oxygen atoms and corresponding O···O distances between Asp25, Asp25′, and darunavir remain essentially unchanged between the two neutron structures, and are also very similar to those in the HIV-1 protease-amprenavir complex (Figure 3; Supporting Information, Figure S2).[34] Consequently, our neutron structures show that proton positions and hydrogen bonding interactions cannot always be deduced correctly merely on the basis of O···O distances determined from single-crystal X-ray structures. Indeed, the positions of deuterium atoms in these structures differ from the hydrogen atom locations inferred by the atomic-resolution X-ray structures of the protease V82A mutant, in complex with a peptidic inhibitor or with darunavir.[36,37] In those X-ray structures, it was incorrectly proposed that H^+ ions were observed in the catalytic site. Reports describing H^+ ions in ultra-high resolution X-ray structures of proteins were recently published,[38,39] but those claims should be treated with extreme caution because H^+, with virtually no electron density around it, should not be visible in electron density maps.

To obtain further insight into the effect of the change in pH on the active site proton transfers, we computed potential energy profiles using a QM/MM approach on models derived from the two neutron structures. Calculations on the pH 6.0 model favor a configuration in which the inner Asp25′ oxygen is protonated and the darunavir hydroxy group is hydrogen bonded to the outer Asp25 oxygen (reactant, R; Supporting Information, Figure S5A). Concerted proton transfers to their respective hydrogen bonded partners indicate a low barrier of 5.0 kcal mol^{-1} (Supporting Information, Figure S5A). A low potential energy barrier, with the inclusion of zero-point energy[33] of about 2 kcal mol^{-1}, is consistent with the observation from neutron crystallography of a shared proton between Asp25′ and darunavir. When the Asp25′ proton was instead placed on the outer oxygen of Asp25′, it transferred spontaneously to the darunavir hydroxy group, the proton of which transfers to the Asp25 inner oxygen, resulting in a configuration resembling the amprenavir-bound structure (Supporting Information, Figure S2).[34] The favored pH 6.0 configuration also predominated in a short QM/MM molecular dynamics simulation, though the trajectory also sampled geometries consistent with the Asp25′ proton shared with darunavir (Supporting Information, Figure S5B). In contrast to the calculations on the pH 6.0 model, analogous calculations on the pH 4.3 model indicated that a stable minimum is reached when the outer Asp25′ oxygen is protonated and the darunavir hydroxy group is hydrogen bonded to the outer Asp25 oxygen (reactant, R; Supporting Information, Figure S5C). Calculated concerted proton transfers to their respective hydrogen bonded partners to obtain a pH 4.3 neutron structure-like configuration indicates a barrier of about 4 kcal mol^{-1} and a stable minimum for the pH 4.3 neutron structure-like configuration (Supporting Information, Figure S5C and S5D). Overall, the calculations support the observations from neutron crystallography and are consistent with the proton transfers triggered primarily by a change in long-range electrostatics from the change in protonation states of the four surface residues.

Herein, we have directly observed induction of changes in hydrogen atom positions in the catalytic site of an enzyme by protonation of distant surface residues. This two-proton transfer in HIV-1 protease results in a proton shift from the inner to the outer carboxylic oxygen atoms on the second subunit of the catalytic Asp dyad, a configuration that is essential for catalysis[29,31,32] and is apparently stable only when surface residues Asp30, Asp30′, Glu34, and Glu34′ are protonated (Supporting Information, Figure S6). Protonation of the surface residues may be vital to the enzyme catalytic cycle, including its maturation from the precursor Gag-Pol.[40,41] In summary, we have demonstrated that neutron crystallography can reveal the positions of protons before and after transfer in biological systems, thus opening up a new field of high-precision mechanistic analysis of biomacromolecular functions.

Experimental Section

Experimental details are provided in the Supporting Information. Coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 5E5J for the HIV-1 triple mutant/darunavir complex at pH 6.0 and 5E5K for the complex at pH 4.3.

Acknowledgements

We thank the Center for Structural Molecular Biology (CSMB) at BES Scientific User Facilities Division, supported by the DOE Office of Biological and Environmental Research (BER), for facility use. O.G., P.L., and A.K. were partly supported by the DOE Office of Basic Energy Sciences (BES). K.L.W. was partly supported by CSMB. I.T.W. was partly supported by an NIH grant R01GM02920. The authors thank Institut Laue Langevin (beamline LADI-III) and Oak Ridge National Laboratory (IMAGINE beamline) for neutron beam time.

Keywords: aspartic protease · enzymes · neutron crystallography · proton transfer · QM/MM modeling

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 4924–4927
Angew. Chem. **2016**, *128*, 5008–5011

[1] R. Kluger in *Enzyme Chemistry* (Ed.: C. J. Suckling), Chapman & Hall, London, **1984**, pp. 8–39.
[2] K. Munson, R. Garcia, G. Sachs, *Biochemistry* **2005**, *44*, 5267–5284.
[3] C. von Ballmoos, P. Dimroth, *Biochemistry* **2007**, *46*, 11800–11809.
[4] M. Morii, M. Yamauchi, T. Ichikawa, T. Fujii, Y. Takahashi, S. Asano, N. Takeguchi, H. Sakai, *J. Biol. Chem.* **2008**, *283*, 16876–16884.
[5] E. Padan, A. Rimon, T. Tzubery, M. Mueller, K. Herz, L. Galili in *Sodium-Hydrogen Exchanger* (Eds.: M. Karmazyn, M. Avkiran, L. Fliegel), Kluwer Academic Publishers, Norwell, MA, **2003**, pp. 91–108.
[6] C. J. T. Grotthuss, *Ann. Chim.* **1806**, *LVIII*, 54–74.
[7] D. Marx, M. E. Tuckermann, J. Hutter, M. Parrinello, *Nature* **1999**, *397*, 601–604.
[8] M. Rini, B.-Z. Magnes, E. Pines, E. T. J. Nibbering, *Science* **2003**, *301*, 349–352.
[9] O. F. Mohammed, D. Pines, J. Dreyer, E. Pines, E. T. J. Nibbering, *Science* **2005**, *310*, 83–86.
[10] R. L. A. Timmer, M. J. Cox, H. J. Bakker, *J. Phys. Chem. A* **2010**, *114*, 2091–2101.
[11] B. Cohen, C. M. Alvarez, N. A. Carmona, J. A. Organero, A. Douhal, *J. Phys. Chem. B* **2011**, *115*, 7637–7647.
[12] D. Stoner-Ma, A. A. Jaye, P. Matousek, M. Towrie, S. R. Meech, P. J. Tonge, *J. Am. Chem. Soc.* **2005**, *127*, 2864–2865.
[13] M. Kaucikas, M. Tros, J. J. van Thor, *J. Phys. Chem. B* **2015**, *119*, 2350–2362.
[14] S. P. Laptenok, A. Lukacs, A. Gil, R. Brust, I. V. Sazanovich, G. M. Greetham, P. J. Tonge, S. R. Meech, *Angew. Chem. Int. Ed.* **2015**, *54*, 9303–9307; *Angew. Chem.* **2015**, *127*, 9435–9439.
[15] M. P. Blakeley, S. S. Hasnain, S. V. Antonyuk, *IUCR J.* **2015**, *2*, 464–474.
[16] A. Shinobu, G. J. Palm, A. J. Schierbeek, N. Agmon, *J. Am. Chem. Soc.* **2010**, *132*, 11093–11102.
[17] A. Y. Kovalevsky, L. Hanson, S. Z. Fisher, M. Mustyakimov, S. A. Mason, V. T. Forsyth, M. P. Blakeley, D. A. Keen, T. Wagner, H. L. Carrell, A. K. Katz, J. P. Glusker, P. Langan, *Structure* **2010**, *18*, 688–699.
[18] C. M. Casadei, A. Gumiero, C. L. Metcalfe, E. J. Murphy, J. Basran, M. G. Concilio, S. C. M. Teixeira, T. E. Schrader, A. J. Fielding, A. Ostermann, M. P. Blakeley, E. L. Raven, P. C. E. Moody, *Science* **2014**, *345*, 193–197.
[19] Q. Wan, B. C. Bennett, M. A. Wilson, A. Kovalevsky, P. Langan, E. E. Howell, C. Dealwis, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 18225–18230.
[20] B. Bennett, P. Langan, L. Coates, M. Mustyakimov, B. Schoenborn, E. E. Howell, C. Dealwis, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18493–18498.
[21] S. J. Tomanicek, R. F. Standaert, K. L. Weiss, A. Ostermann, T. E. Schrader, J. D. Ng, L. Coates, *J. Biol. Chem.* **2013**, *288*, 4715–4722.
[22] 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.
[23] 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.
[24] 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.
[25] S. Yamaguchi, H. Kamikubo, K. Kurihara, R. Kuroki, N. Niimura, N. Shimizu, Y. Yamazaki, M. Kataoka, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 440–444.
[26] N. Niimura, A. Podjarny, *Neutron Protein Crystallography*, Oxford, New York, NY, **2011**, p. 232.
[27] I. T. Weber, Y.-F. Wang in *Aspartic Proteases as Therapeutic Targets. Methods and Principles in Medicinal Chemistry*, *Vol. 45* (Ed. A. K. Ghosh), Wiley-VCH, Weinheim, **2010**, pp. 109–137.
[28] I. T. Weber, D. W. Kneller, A. Wong-Sam, *Future Med. Chem.* **2015**, *7*, 1023–1038.
[29] S. Bjelic, J. Åqvist, *Biochemistry* **2006**, *45*, 7709–7723.
[30] V. Y. Torbeev, S. B. Kent, *Org. Biomol. Chem.* **2012**, *10*, 5887–5891.
[31] V. Y. Torbeev, H. Raghuraman, D. Hamelberg, M. Tonelli, W. M. Westler, E. Perozo, S. B. Kent, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 20982–20987.
[32] S. Piana, D. Bucher, P. Carloni, U. Rothlisberger, *J. Phys. Chem. B* **2004**, *108*, 11139–11149.
[33] M. A. Porter, P. A. Molina, *J. Chem. Theory Comput.* **2006**, *2*, 1675–1684.
[34] I. T. Weber, M. J. Waltman, M. Mustyakimov, M. P. Blakeley, D. A. Keen, A. K. Ghosh, P. Langan, A. Y. Kovalevsky, *J. Med. Chem.* **2013**, *56*, 5631–5635.
[35] M. Adachi, T. Ohhara, K. Kurihara, T. Tamada, E. Honjo, N. Okazaki, S. Arai, Y. Shoyama, K. Kimura, H. Matsumura, S. Sugiyama, H. Adachi, K. Takano, Y. Mori, K. Hidaka, T. Kimura, Y. Hayashi, Y. Kiso, R. Kuroki, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4641–4646.
[36] J. Brynda, P. Rezacova, M. Fabry, M. Horejsi, R. Stouracova, J. Sedlacek, M. Soucek, M. Hradilek, M. Lepsik, J. Konvalinka, *J. Med. Chem.* **2004**, *47*, 2030–2036.
[37] Y. Tie, P. I. Boross, Y.-F. Wang, L. Gaddis, A. K. Hussain, S. Leshchenko, A. K. Ghosh, J. M. Louis, R. W. Harrison, I. T. Weber, *J. Mol. Biol.* **2004**, *338*, 341–352.
[38] H. Ogata, K. Nishikawa, W. Lubitz, *Nature* **2015**, *520*, 571–574.
[39] D. A. Nichols, J. C. Hargis, R. Sanishvili, P. Jaishankar, K. Defrees, E. W. Smith, K. K. Wang, F. Prati, A. R. Renslo, H. L. Woodcock, Y. Chen, *J. Am. Chem. Soc.* **2015**, *137*, 8086–8095.
[40] L. J. Hyland, T. A. Tomaszek, Jr., T. D. Meek, *Biochemistry* **1991**, *30*, 8454–8463.
[41] J. M. Louis, R. Ishima, D. A. Torchia, I. T. Weber, *Adv. Pharmacol.* **2007**, *55*, 261–298.

Received: October 26, 2015
Revised: January 27, 2016
Published online: March 9, 2016

