X-ray/Neutron Crystallography

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Identification of the Elusive Hydronium Ion Exchanging Roles with a Proton in an Enzyme at Lower pH Values**

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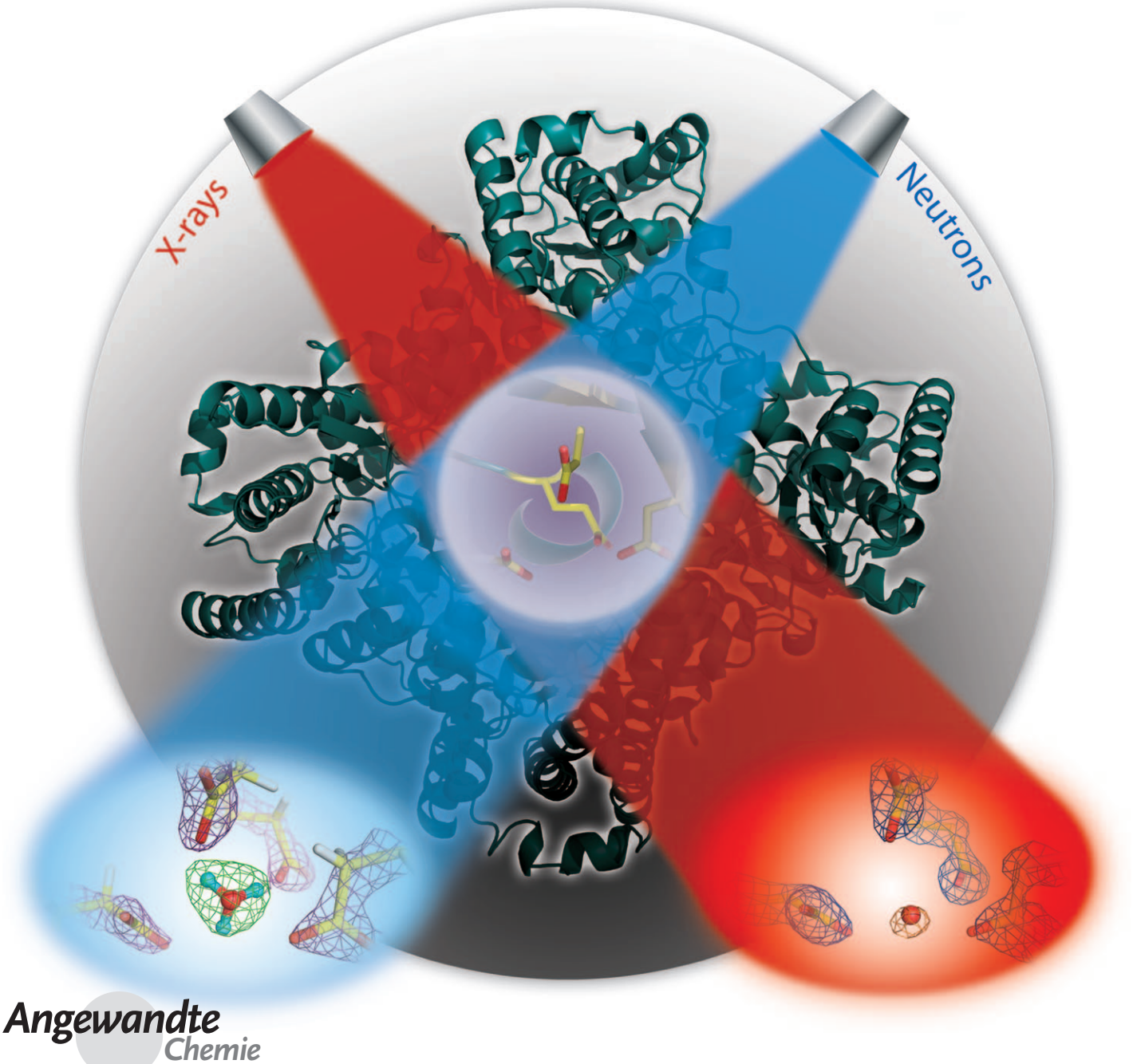

Hydrogen ions (protons, H^+) and hydrated protons, hydronium ions (H_3O^+), are thought to be crucial players in many chemical and biological processes. Despite their proposed importance, hydronium ions have never been identified in the crystallographic structures of biological macromolecules. These elusive molecules are thought to take part in the transfer of protons during enzyme-catalyzed reactions and also membrane translocations by proton pumps. For example, experimental data and simulations of the proton pumps H^+,K^+-ATPase and ATP synthase have suggested hydronium ions bind to specific sites at basic pH values.[1–3] The problem is that hydronium ions and water molecules appear as similar peaks at the locations of oxygen atoms in electron-density maps calculated from X-ray diffraction data, so it is impossible to tell a water molecule apart from a hydronium ion.

X-rays are scattered by electrons; therefore, hydrogen, the smallest of atoms with just one electron, is difficult to see. Indeed, electron-bare protons are completely invisible. Neutrons, on the other hand, are scattered by atomic nuclei, and neutrons can be used to visualize hydrogen atoms and protons and thus to distinguish between hydronium and water molecules, especially when hydrogen has been replaced by its strongly scattering isotope deuterium. We have been exploiting the ability of neutrons to "see" protons in our studies of the catalytic mechanisms of enzymes.[4] Having used neutrons to provide new insight into its catalytic mechanism,[5] we recently focused our attention on trying to understand why the activity of the enzyme D-xylose isomerase (XI) is so sensitive to the pH value.

One reason for our interest in this enzyme is that it could enable more efficient conversion of woody biomass into value-added bioproducts by isomerizing the sugar D-xylose into its fermentable keto isomer D-xylulose. However, the activity of the enzyme is highest at pH ≈ 8, and under the often acidic (pH < 6) conditions of biomass conversion, the metal cofactors required for isomerization are expelled from their two binding sites (M1 and M2) in the active site.[6] At pH 7.7, the metal cofactor at binding site M1 is coordinated by the side chains of amino acid residues Asp245, Glu217, Glu181, and Asp287 and two water molecules, in an octahedral arrangement.[7] We stripped the enzyme of its metal cofactors with a strong chelating agent and crystallized it at pH 5.9 and 7.7. After soaking the crystals in D_2O to replace labile hydrogen atoms with deuterium atoms, we collected both neutron and X-ray crystallographic data for use in a joint X-ray/neutron structure-refinement process.[8]

We found that at pH 7.7, when the metal is absent from M1, its place is taken by a hydronium ion, which is observed as D_3O^+ (Figure 1 a). The hydronium ion seems to template the shape and charge of this M1 site for metal binding. The

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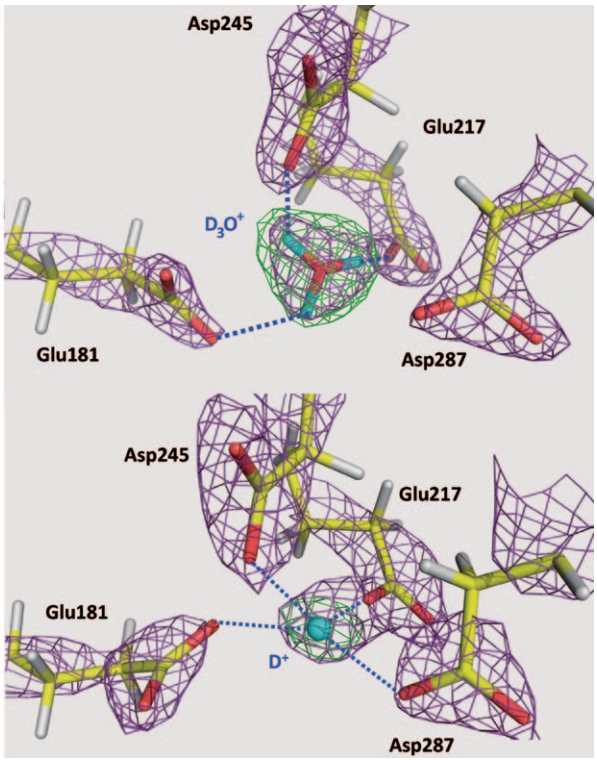

Figure 1. The M1 metal-binding site of the D-xylose isomerase active site: $2F_O-F_C$ (violet) and omit F_O-F_C (green) positive neutron scattering density maps of the active site of D-xylose isomerase showing a) D_3O^+ at pH 7.7 and b) D^+ at pH 5.9; H bonds are shown as blue dotted lines. Coordinates and structure factors have been deposited in the Protein Data Bank with the accession codes 3KCJ (apo-XI, pH 7.7), 3QZA (apo-XI, pH 5.9), and 3QYS (XI–0.6 Ni^{2+} complex, pH 5.8 (see text)).

identification of the hydronium ion is unequivocal because of the shape and occupancy of its associated peaks in the neutron and X-ray scattering density maps. The omit F_O-F_C neutron scattering density map for D_3O^+ resembles a trigonal pyramid. Contoured at a 4σ level, this feature is the largest in the difference map. Thus, the omit map shape eliminates the possibility that this entity could be a deuterated ammonium cation (ND_4^+) that was present in the crystallization medium. The occupancies for all atoms refined to 1.0, which provides evidence that the species contains three D atoms connected to an oxygen atom. If it were a disordered D_2O molecule, the occupancies for two of the three D atoms should have refined to about 0.5. We also assured ourselves that no significant amount of metal was left by using inductively coupled plasma mass spectrometry (ICP-MS) on crystals that had been dissolved after crystallographic data collection. The ICP-MS spectra showed that the total quantity of metals, such as Mg^{2+}, Zn^{2+}, and Cu^{2+}, that remained at M1 and M2 was low enough (< 5%) that these metals would not be detected with X-rays. D-Xylose isomerase has the ability to scavenge alkali-earth and heavy metals with micromolar affinity; the low levels of metals detected by ICP-MS were probably scavenged from minute quantities (< 5 ppm) in the crystallization buffers and salts.[9]

At pH 5.9, the hydronium ion at the M1 binding site is dehydrated to a proton (D^+) in a trifurcated low-barrier hydrogen bond, in which the proton is positioned closest (1.3 Å) to, and thus donated by, Glu217 (Figure 1b). This time, identification as a proton is unambiguous because of the occupancy and location of the peak, contoured at a 4σ level, in the neutron scattering density map and its absence in the electron density map. The amino acid residues have collapsed around the proton, and the site no longer has the required shape to accept a metal cation (Figure 2). We tried to force metals to bind at the protonated M1 site in crystals of the enzyme at pH 5.8 by soaking the crystals in highly concentrated solutions of nickel salts. However, X-ray crystallographic studies showed that although the M2 site could be occupied by a metal up to a level of 60%, the protonated M1 site remained without a metal and remained collapsed around a proton with the carboxylic side chains of residues 181, 217, 245, and 287 in the same arrangement as in the low-pH-value metal-free structure.

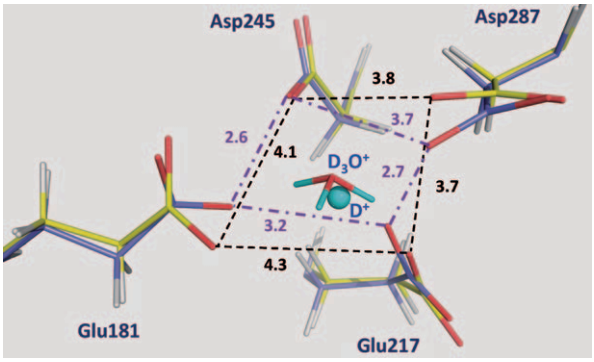

Figure 2. Superposition of the M1 metal-binding sites of the structures of the apo form of D-xylose isomerase at the pH values of 7.7 (yellow carbon atoms) and 5.9 (blue carbon atoms) showing the collapse of side chains in this site by more than 1 Å in terms of the differences in O···O separation. These structural changes are significant considering the coordinate errors (diffraction-data precision indicator, DPI) in both structures of 0.18 Å.

These observations provide an explanation for why the required cofactor metal cations are not bound, and therefore why there is a dramatic decrease in the activity of the enzyme at low pH values. More importantly, the crystallographic experiments enabled the first direct visualization of hydronium ion in a biological system and provided evidence for its interchangeability with a proton at lower pH values. Furthermore, proton chelation by oxygen-based ligands has not been observed previously in a biomacromolecular system; such interactions have previously been detected and studied in small molecules.[10] Our results have broad implications for the possible role of hydronium ions in other biological systems. In particular, the proton pump gastric H^+,K^+-ATPase, an important drug target in the treatment of peptic ulcer and gastroesophageal reflux diseases,[11] and also ATP synthase,[1] which together with H^+,K^+-ATPase is responsible for the synthesis and hydrolysis of adenosine triphosphate (ATP), have glutamic and aspartic acid rich clusters, like the metal-binding site in D-xylose isomerase, and may bind hydronium as a key step in their functions. Hydronium ions may also be involved in other processes, such as the transport of sugar by lactose permease.[12] It may be that as the field of neutron protein crystallography continues to grow,[4] other exotic molecular species will be found in biological systems.

Experimental Section

D-Xylose isomerase (XI, MW = 172 kDa) was purchased from Hampton Research Corp. (Aliso Viejo, CA). Samples were prepared and crystallized as described previously.[5,7] Crystals were mounted in quartz capillaries containing buffers made with 99.9% D_2O.

X-ray crystallography: X-ray crystallographic data were collected at room temperature on a Rigaku FR-E instrument with an R-Axis IV++ detector to 2, 1.7, and 1.85 Å resolution for the metal-free crystals grown at pH 7.7 and 5.9 and for the crystal containing 60% Ni^{2+} at the M2 metal-binding site at pH 5.8, respectively, with $Cu_{K\alpha}$ radiation ($\lambda = 1.5418$ Å). Diffraction data were integrated and scaled by using the CrystalClear/d*TREK software,[13] and the structures were refined by using SHELX.[14] For joint X-ray/neutron crystal-structure refinement, the X-ray crystallographic data were collected from crystals taken from the same crystallization drops that provided the larger crystals for neutron data sets.

Neutron crystallography: Monochromatic neutron crystallographic data were collected from the metal-free XI crystal (pH 7.7) to 1.8 Å resolution at room temperature on the instrument D19 at the Institut Laue Langevin (ILL, Grenoble, France) with a wavelength of 2.422 Å over a period of six days. The faces of the crystal ($5.5 \times 2.5 \times 2$ mm^3, 28 mm^3) were carefully indexed to enable an absorption and attenuation correction to be applied to the measured intensities, with transmission values T_{min} and T_{max} of 0.37 and 0.60, respectively. The data were processed with the ILL program RETREAT,[15] corrected for effective absorption, and then merged with SCALA from the CCP4i suite of programs.[16,17] Time-of-flight wavelength-resolved Laue images were collected at room temperature at the neutron Protein Crystallography Station (PCS) at Los Alamos Neutron Science Center (LANL, Los Alamos, USA) on a Huber κ-circle goniometer for the metal-free XI crystal (pH 5.9, $2.5 \times 2.5 \times 1.5$ mm^3, 9 mm^3) to 2.0 Å resolution over a period of 14 days.[18] Each image was processed by using a version of d*TREK modified for wave-

length-resolved Laue neutron protein crystallography.[13] The integrated reflections were wavelength normalized by using LAUENORM[19] and then merged by using SCALA incorporated into the CCP4i suite of programs.

Joint XN structure determination: The joint X-ray/neutron (XN) crystal structures of metal-free apo-XI were determined by using nCNS.[8] Rigid-body refinement was followed by several macrocycles of refinement of positional and atomic displacement, followed by water building. The D_2O orientations were established on the basis of the $2F_O-F_C$ and F_O-F_C neutron scattering density maps and by also taking into account potential hydrogen bonding. The level of H/D exchange at labile positions was refined in the last stages of the joint refinement.

ICP-MS: Mass spectrometry analysis was performed on crystals used in neutron-diffraction experiments. The data were collected from solution samples by dissolving the crystals and detecting the amounts of various metals present. The data showed negligible (< 5%) total quantities of metals able to bind to the two metal-cofactor binding sites in each crystal; metals in such small quantities are undetectable with X-rays.

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