than the previous state of the art.[2] As a demonstration, they imaged a single organic molecule, porphyrin, illustrated on page 15.

In the experiment, both tip and surface were composed of silver, a metal thought to produce the strongest enhancements to a Raman signal. The coupling between the tip and surface in a light field drives the electrons back and forth and boosts the signal enhancement—particularly when they are driven at their natural resonance frequency.

The team illuminated the tip with green laser light and monitored the Raman-shifted red light backscattered from the porphyrin. Each of the scattered light's Raman peaks signifies one of many vibrational modes. Subtle variations in the detected intensity of one of those modes (920 cm^{-1}) produced the ring-shaped image (top left in the figure) as the tip passed over different parts of the molecule. That contrast agrees well with the simulated signal (bottom left).

Dong and colleagues also demonstrated how the molecule's full set of vibrational modes is influenced by its orientation. As the bonding configurations between molecule and silver surface differed—with the porphyrin adsorbed flat onto a silver terrace, say, or at an angle on a silver step edge—so did the position of the Raman peaks. To see how much, the researchers positioned the tip over identical molecular features for side-by-side comparison of the spectra.

The system's low temperature boosted the signal-to-noise ratio by suppressing the molecule's diffusion and desorption, and its ultrahigh vacuum environment preserved a pristine substrate and stabilized the tip within a nanometer or so above a molecule. Those conditions, says Dong, allowed the team to precisely tune the plasmon resonance frequency to match that of the Raman emission for maximum enhancement.

Locating and controlling the plasmon frequency has traditionally been difficult. To find it, the researchers developed a clever approach: lower the tip to a molecule and raise the STM tunneling current until the porphyrin begins to glow; conveniently, the luminescence serves as a diagnostic for the plasmon resonance. And because the resonance depends not just on the tip's composition but also on its size and shape, the researchers found they could sharpen the tip *in situ*, using an ion sputtering gun, and monitor the changing sample luminescence until its broad envelope overlapped both the incident laser frequency and specific Raman emissions.

When the resonances matched, the TERS signal rose. What's more, it did so nonlinearly with incident laser power. The nonlinearity may suggest that some new, unconventional TERS mechanism is at play. "Unlike conventional TERS, in which the intensities of incident and Raman-shifted photons are linearly related, some kind of higher-order Raman process may confine the tip–sample interaction more tightly," speculates Northwestern University's Richard Van Duyne.

Details of the mechanism remain unclear. But the implications of pinning them down are broad: Catalysis, photochemistry, DNA sequencing, and protein folding, all imaged at the single-molecule scale, are just a few applications in a very long list.

Mark Wilson

References

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2. See, for example, R. Treffer et al., *Biochem. Soc. Trans.* **40**, 609 (2012); T. Ichimura et al., *Phys. Rev. Lett.* **102**, 186101 (2009).

X-ray diffraction details water's path through a cell pore

A high-resolution crystallographic structure reveals why aquaporin proteins are permeable to water but not to protons.

Certain biological processes—producing tears and processing urine, for instance—require cells to expel or take in water faster than it can diffuse through a cell membrane. In those cases, cells deploy aquaporins, special proteins that straddle a cell membrane and form water-permeable pores. Discovered two decades ago by Peter Agre (see PHYSICS TODAY, December 2003, page 27), aquaporins are now known to provide the biomolecular plumbing for numerous species of plants, animals, and bacteria.

The proteins are highly selective gatekeepers. All of the dozens of known variants contain a narrow passageway—a so-called selectivity filter (SF), positioned near the pore's extracellular opening—that stems the flow of large solutes. Likewise, strategically positioned charge centers embedded in the pore wall create potential barriers that block the flow of ions.

Harder to explain, however, is aquaporins' impermeability to protons. Because protons can hop freely along a network of hydrogen-bonded water molecules, one would expect a water-filled pore to conduct protons in much the same way that a wire conducts electrons—and that would make it impossible for a cell to preserve the transmembrane potentials needed to power cellular machinery.

Presumably, aquaporins possess some structural feature that precludes the formation of a pore-spanning hydrogen-bond network. In one theory, now more than a decade old, the key feature is a positively charged NPA constriction—so called because it's lined with asparagine (N), proline (P), and alanine (A) amino acids. Located at the pore's midpoint, the NPA constriction generates an electric field thought to configure nearby water molecules so that their oxygen atoms face one another—an orientation ill suited to hydrogen-bond formation.

Starting around 2006, a series of experiments by Eric Beitz (University of Kiel, Germany) and collaborators began to cast doubt on that prevailing thinking.[1] The researchers generated mutant aquaporins having a charge-neutral NPA constriction, and they found that those pores showed negligible change in their proton permeability. When they instead modified amino acids in the SF, protons began to seep through.

Now a collaboration led by Richard Neutze (University of Gothenburg, Sweden) and Emad Tajkhorshid (University of Illinois at Urbana-Champaign) has used x-ray crystallography to construct the most detailed picture to date of the inner workings of an aquaporin protein.[2] And that picture seems to confirm at least a partial role for the SF in aquaporin's proton blockade.

Bond hunting

Membrane proteins are notoriously difficult to crystallize and typically yield x-ray structures with resolutions of no better than 2 Å or so. Working with a yeast aquaporin that proved exception-

ally amenable to crystallization, however, Gerhard Fischer, a graduate student in Neutze's lab, was able to grow crystals large enough and uniform enough to diffract at 0.88-Å resolution—a record for a membrane protein.

The diffraction data yielded a detailed electron-density map of the inner region of the pore, as shown in the figure below. Gray mesh contours indicate electron clouds of the protein's atoms; orange contours indicate electron clouds of water molecules occupying energetically favorable sites inside the pore. During transport, water molecules traverse the pore in single file by hopping from one site to the next.

To reconstruct a hydrogen-bond network, one needs to know which so-called donor atoms (always hydrogen) interact with which acceptors (oxygen, nitrogen, or some other highly electronegative atom). But hydrogen atoms—whether in the protein or in water—lack sufficient electron density to produce distinct x-ray diffraction peaks and therefore don't appear in the electron-density map. To clearly see them, Neutze and coworkers constructed what's known as a hydrogen omit map, a map of the difference between the system's measured electron density and the expected electron density if the hydrogen atoms were removed. In such a map, hydrogen atoms appear as regions of excess measured density. In the inset, excess density corresponding to the protein's hydrogen atoms is shown in green. (Water's hydrogen atoms aren't visible.)

Omit maps aren't new to protein crystallography, but Neutze and coworkers are the first to generate one detailed enough to see an aquaporin's hydrogen atoms individually. That allowed the researchers to determine which atoms embedded in the pore wall were potential hydrogen-bond acceptors and which were potential donors. The detected structural arrangement of those acceptors and donors suggests that water molecules in the SF are likely to form hydrogen bonds with atoms in the pore wall rather than with one other.

The inset, for example, indicates two hydrogen bonds lying between a water molecule in the SF and sites on histidine and arginine amino acids that line the pore wall. A third bond between the molecule and the pore lies normal to the page and isn't shown. Because water can form at most four hydrogen bonds, the molecule can't participate in enough water–water bonds to propagate a chain. Other water molecules in the SF are similarly tied up by the pore, and the result is a fragmented hydrogen-bond network. Comments Beitz, "This will likely bring to an end a 10-year discussion in the aquaporin community. It's now quite clear that both the NPA constriction and the SF contribute to blocking protons."

The team's x-ray structure also yielded a surprise discovery: The four water sites in the SF are spaced so closely—roughly 1.5 Å, about 1 Å less than the preferred water-molecule spacing—that no two neighboring sites can be occupied simultaneously. Molecular dynamics simulations by collaborators Tajkhorshid and Giray Enkavi show that pairs of water molecules move between those sites in highly coordinated fashion: Molecules in the first and third positions jump in unison to the second and fourth positions. The finding points to a possible evolutionary link with potassium ion channels, which convey potassium ions in similar fashion.

Ashley G. Smart

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An electron-density map of a yeast aquaporin shows two structural motifs—the selectivity filter and the so-called NPA constriction—thought to factor into the pore's impermeability to protons. Gray mesh contours show the electron clouds of the protein's carbon (gray), nitrogen (blue), oxygen (red), and sulfur (yellow) atoms; orange contours show the electron clouds of water molecules inside the pore. In the inset, a so-called hydrogen omit map of the selectivity filter, green contours correspond to hydrogen atoms on the protein. The atoms' arrangement suggests multiple water–pore hydrogen bonds, including the two indicated here by the dashed lines. (Adapted from ref. 2.)