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Directional Proton Transfer in Membrane Proteins Achieved through Protonated Protein-Bound Water Molecules: A Proton Diode**

*Steffen Wolf, Erik Freier, Meike Potschies, Eckhard Hofmann, and Klaus Gerwert**

Dedicated to Professor Manfred Eigen

The key function of energy-transducing membrane proteins is the creation of a proton gradient by directional proton transfer. The role of protein-bound water molecules herein is not fully understood, as X-ray diffraction analysis has resolved the positions of oxygen, but not of hydrogen atoms in such protein–water complexes. Here we show, now time-resolved at atomic resolution, how a membrane protein achieves directional proton transfer via protein-bound water molecules in contrast to random proton transfer in liquid water. A combination of X-ray structure analysis, time-resolved FTIR spectroscopy, and molecular dynamics (MD) simulations elucidates how directionality is achieved. Using the proton-pump bacteriorhodopsin as the paradigm, we show how controlled conformational changes of few amino acid residues rearrange preordered water molecules and induce directional proton transfer. This mechanism is analogous to an electronic diode: a "proton diode".

According to the chemiosmotic theory, the creation of a proton gradient in photosynthesis[1] and oxidative phosphorylation[2–4] by means of directional proton transfer is the key step for energy transduction in living cells. ATPases use this proton gradient to produce ATP, the fuel for life. In contrast to this directional mechanism in proteins, proton transfer in liquid water is random.[5,6] Bacteriorhodopsin (bR), a protein that belongs to the microbial rhodopsin family,[7,8] achieves this directional proton transfer by a light-driven proton-pumping mechanism. Like other microbial rhodopsins, bR exhibits a structural motif of seven transmembrane α-helices and a retinal chromophore covalently bound to a lysine through a protonated Schiff base. The light-induced retinal isomerization from all-*trans* in the ground state (BR) to the 13-*cis* conformer drives bR through a photocycle with intermediates named J, K, L, M, N, and O in order of their appearance.[8]

During the L to M transition, the protonated Schiff base ($C{=}NH^+$), the central proton-binding site, deprotonates and protonates its counterion Asp85[9] (step 1 in Figure 1a). Protonation of Asp85 breaks its salt bridge to Arg82, which then moves towards Glu194/Glu204 (step 2). The orientation of Arg82 depends on the protonation state of Asp85.[10] The arginine movement destabilizes a protonated water cluster between Arg82, Glu194, and Glu204 (step 3 in Figure 1a), and a proton is released to the bulk.[11,12]

However, the detailed nature of the proton-release group is still under debate. QM/MM simulations of the proton-release group propose a shared proton between Glu194 and Glu204,[13] a Zundel cation with two water molecules ($H_5O_2^+$),[14] or an asymmetric Eigen cation of four water molecules ($H_9O_4^+$).[15] From time-resolved FTIR experiments with site-directed mutations around the protonated water cluster and H/D-exchange experiments we have concluded that the proton-release group forms a protonated water cluster, most likely an asymmetric Eigen ion as shown in Figure 1a in purple.[12] Glu194 and Glu204 are clearly deprotonated in the bR ground state.[11] This experimental result was recently confirmed by Lórenz-Fonfría et al.[16] Nevertheless, the exact nature of the protonated water cluster and the release mechanism has still to be determined.

Here, we used X-ray structure analysis to determine the positions of the water oxygen atoms and FTIR difference spectroscopy to determine the dynamics of the corresponding water hydrogen atoms. The proton release to the bulk in the L

[*] Dr. S. Wolf,[+] E. Freier,[+] Dr. M. Potschies,[$] Prof. Dr. E. Hofmann, Prof. Dr. K. Gerwert
Lehrstuhl für Biophysik, Ruhr-University Bochum
Universitätsstrasse 150, 44780 Bochum (Germany)
Fax: (+49) 234-321-4238
E-mail: gerwert@bph.rub.de
Homepage: http://www.bph.rub.de
Dr. S. Wolf,[+] Prof. Dr. K. Gerwert
Department of Biophysics
CAS–Max-Planck Partner Institute for Computational Biology
Shanghai Institutes for Biological Sciences
320 Yue Yang Road, 200031 Shanghai (P.R. China)
[$] Current address:
Lehrstuhl für Biophysik, Universität Konstanz (Germany)
[+] These authors contributed equally to this work.

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Supporting information for this article (including details of protein purification, FTIR measurements, X-ray crystallography, and MD simulations) is available on the WWW under http://dx.doi.org/10.1002/anie.201001243.

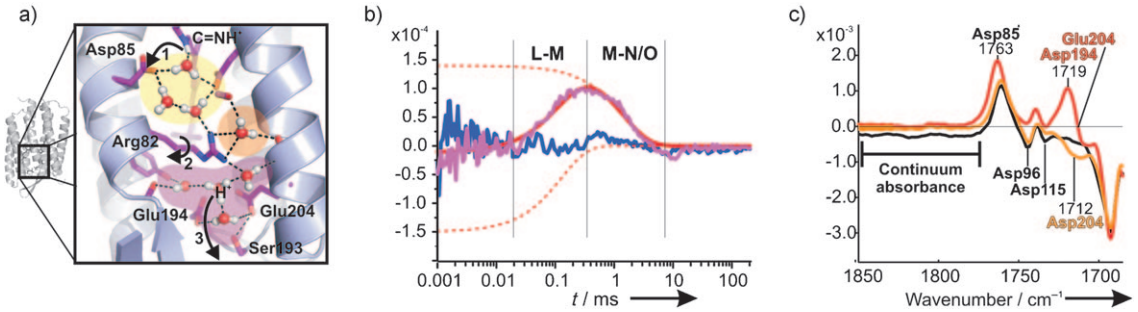

Figure 1. Proton release of bR monitored by time-resolved FTIR spectroscopy (for details see text). a) Proton release mechanism during the L–M transition. b) Time-resolved changes in IR absorbances during the bR photocycle (see the Supporting Information) at pH 7 (blue) and at pH 5 (purple; transiently shared proton of the glutamates 194/204 at 1706–1720 cm^{-1}). c) M-BR difference spectra of wild-type bR (black), E204D (orange), and E194D (red).

to M transition is accomplished within 60 μs at pH 7.[11,17] However, because proton release at pH 5 is delayed,[11,17] we can now resolve a shared proton at Glu194/Glu204 in the L to M transition (at 1706—1720 cm^{-1}), as is evident in Figure 1b and in Figure 1 in the Supporting Information. At pH 7, this transient protonation is not resolved apparently because of kinetic reasons: the deprotonation of the Glu194/Glu204 complex occurs faster than its protonation, therefore no intermediate is accumulated. Here, we have monitored clear-cut a shared proton at Glu194/204 as a transient phenomenon during the photocycle. This shows experimentally that the simulations of Phatak et al.[13] apparently reflects an intermediate state in the release mechanism, but not the ground-state situation. This result could be explained by the fact that these simulations were based on an L intermediate crystal structure[18] (PDB-ID 1UCQ), but not the ground state. The results of QM/MM simulations depend strongly on the underlying 3D structural model.

To achieve further detailed insight into the release mechanism we replaced Glu194 and Glu204 with the respective aspartate residues in mutant bR proteins. Surprisingly, in the E194D mutant the continuum absorbance of the protonated water cluster disappears (Figure 1c, red), indicating the absence of a water cation at the release site in E194D. Instead, Glu204 is protonated in the bR ground state (negative band around 1712 cm^{-1}) in contrast to the wild-type (WT) protein, deprotonates in the L to M transition (negative band at 1712 cm^{-1} in Figure 1c), and protonates Asp194[11] (positive band at 1719 cm^{-1} in Figure 1c). The proton-release mechanism in the mutant E194D is thus different than in the WT. Furthermore, in E204D, the continuum band merely decreases in intensity. Asp204 is protonated in the bR ground state, sharing its proton with the water cluster, and it then deprotonates in M[11] (negative band at 1712 cm^{-1} in Figure 1c).

To elucidate the structural changes underlying the different release mechanisms, we determined the X-ray structures of both bR mutants. Interestingly, the downward movement of Arg82 during the WT photocycle can already be observed sequentially in the ground-state structures of both mutants as given in Figure 2a. In E204D (orange, Figure 2a), a slight shift of Arg82 is observed compared to its position in the 1.55 Å WT protein structure.[19] In E194D (red) the full Arg82 movement is observed and one water molecule is shifted from position c to d. Water position d lies at the border between the protein-internal water cluster and the external protein surface, bridging internal and external water molecules. In E204D, the partial Arg82 movement shifts water position c slightly towards Glu204 and also to position d. We propose that these observations in the X-ray structures of the mutants reflect shifts of the water population in the WT protein from c to d during the photocycle.

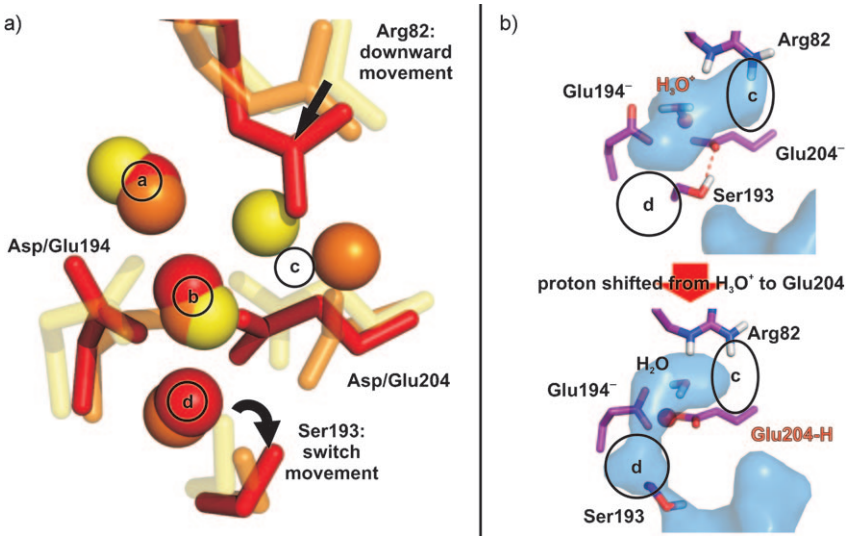

Figure 2. Proton-release site in bR (for details see text). a) Crystal structures of WT bR (yellow, PDB-ID 1C3W,[19] 1.55 Å), the E204D mutant (orange, PDB-ID 2WJK, 2.30 Å), and the E194D mutant (red, PDB-ID 2WJL, 2.15 Å). The positions of the water oxygen atoms are shown as spheres. b) The space occupied by water molecule dynamics,[20,21] shown as a Connolly surface in blue, as revealed by MD simulations.

The arginine downward movement disturbs the stabilizing second hydration shell of the protonated water cluster. In the Grotthuss proton-transfer mechanism in water and ice through the interplay of Eigen ($H_9O_4^+$) and Zundel ($H_5O_2^+$) cations,[6] it is not the fast hopping of the proton between water molecules that limits proton transfer rates, but the slower cleavage of a hydrogen bond to a water molecule in the second hydration shell. In liquid water, these changes in hydrogen bonds of the second hydration shell occur randomly and on a picosecond timescale, resulting in random and fast proton transfer. In bacteriorhodopsin, the second hydration shell of randomly moving water molecules is substituted by conformationally fixed amino acids.[11] This mimic of the second hydration shell presents a structurally stable cavity for the protonated water cluster in the bR ground state.[12,22–24] The controlled movement of Arg82 in the "second hydration shell" destabilizes the protonated water cluster in a controlled fashion, and the proton is released.[12] The downward movement of Arg82 seems to shift the protonated water cluster more towards Glu194/204. It may reflect a transition from an asymmetric Eigen complex to a Zundel ion. The distance between the side-chain oxygen atoms of Ser193 and Glu204 increases from 2.6 Å in the wild-type protein, to 3.0 Å in E204D, and 3.2 Å in E194D and therefore represents a weakening of the hydrogen bond during the opening process. We therefore think that the weakening of the Ser193/Glu204 hydrogen bond coincides with the transfer of the proton towards Glu204, most probably because the charges on the Glu204 side-chain oxygen atoms are decreased. It was shown that this hydrogen bond constitutes a gate preventing bulk water from invading the protein and connecting to the internal water molecules.[20] The structural models of the wild-type protein, E204D, and E194D form a set, which enables us to monitor the structural changes during the proton release as in a stepwise movie or a flipbook.

We analyzed furthermore by MD simulations the influence of protonation of the glutamates in the WT protein on the rearrangement of the water molecules. In our MD simulations with an explicit H_3O^+ ion, we found that the distance between the side-chain oxygen atoms of Glu194 and Glu204 of 3.7 Å (Figure 3a, red graphs) is in reasonable agreement with the 3.0 Å in the bR crystal structure 1C3W.[19] In contrast, the simulations by Phatak et al.[13] reported a distance of 5.3 Å for a protonated water molecule. The water arrangement is a trigonal pyramid like that in an Eigen ion, while the hydronium ion itself sits at a corner of the pyramid (Figure 3b, left). It bridges Glu194 and Glu204 by forming hydrogen bonds to both of them. We qualitatively mimicked the situation of a shared proton between Glu194 and Glu204 and the related change of atomic charges of Glu204 by a full protonation of Glu204 from the explicit H_3O^+ ion in the release group. Upon this in silico protonation of Glu204, we see a shift of water molecules from position c to d and a cleavage of the Ser193/Glu204 bond (Figure 2b and Figure 2 in the Supporting Information). This is in nice agreement with the observation of the shift of water molecules and the lengthening of the Ser193/204 bond in the crystal structures of the mutants. The cleavage allows bulk water to merge with the internal water cluster and provides a pathway for the proton to the bulk solvent.

As shown in Figure 2, the rearrangement of water molecules observed in MD simulations matches nicely the one observed in the "flipbook" arrangement of bR mutant structures. The protonated Glu204 forms a stable hydrogen bond to Glu194 with side-chain oxygen distances of 2.6 Å (see Figure 3a, blue graphs). The simulation agrees nicely with those by Phatak et al. for a shared proton (see Figure 2B and D in Ref. [13]: 2.8 and 2.5 Å, respectively). The water molecules now form a linear chain and may serve as a Grotthuss-type conductor to the bulk water (see Figure 3b, right). Interestingly, Phatak et al. saw in their QM/MM simulations an IR band around 1750 cm^{-1} (see Figure 3A in Ref. [13]), which agrees well within the precision of their simulation method[25,26] with our signal at 1720–1706 cm^{-1} for

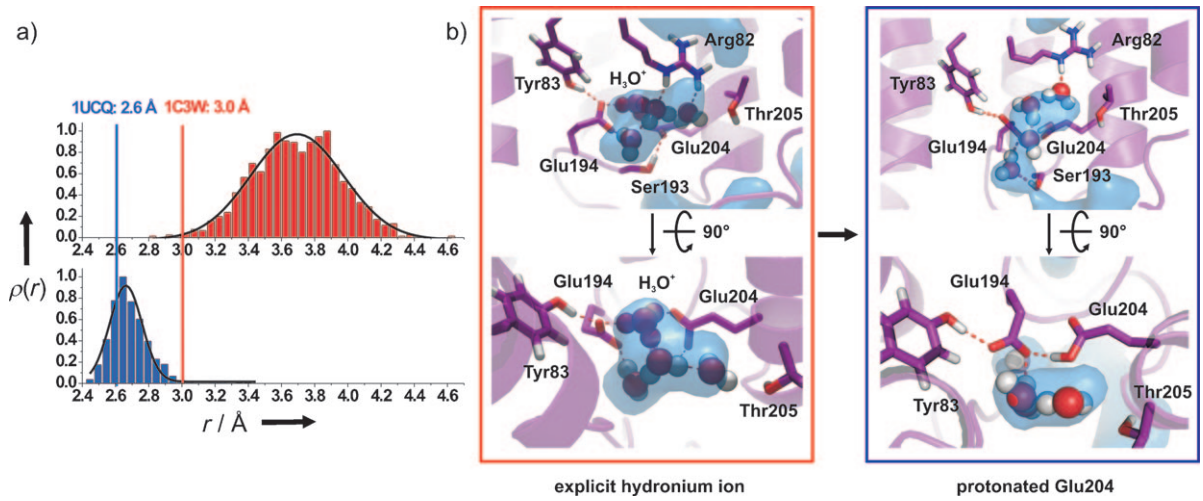

Figure 3. MD simulations of the ground-state structure 1QHJ with explicit hydronium ion (red) and with the "shared-proton" model (blue); for details see text. a) Distances between the side-chain oxygen atoms of Glu194 and Glu204; Gaussian fit of the distance distribution in black. b) Arrangement of the water cluster in the MD simulation within the proton-release site. Water density[20,21] (see Figure 2b) as blue surface, water molecules/hydronium ion as spheres (H_3O^+ in red).

the photocycle intermediate. We want to point out that Phatak et al. used an L intermediate crystal structure (PDB-ID 1UCQ)[18] for their simulations. Their simulations obviously reproduce a state that we observe here during the photocycle. Their conclusion of a shared proton agrees nicely with the intermediate, but not with the ground state as they propose.

Summing up the proton-release mechanism so far: Asp85 is protonated during the L to M transition, inducing the downward movement of Arg82. The protonated water molecules are moved towards Glu194/204, which results in a transient sharing of a proton by Glu194/204. The Ser193/Glu204 gate opens, and internal and external water molecules form contacts and provide an unidirectional proton release pathway. The flow of protons back to the release group is prevented by the low pK_a value of 5.3 of the proton-release group in M.[10] In agreement with this, Lórenz-Fonfría and Kandori found the proton release in the M intermediate to be an irreversible step in the bR photocycle.[27]

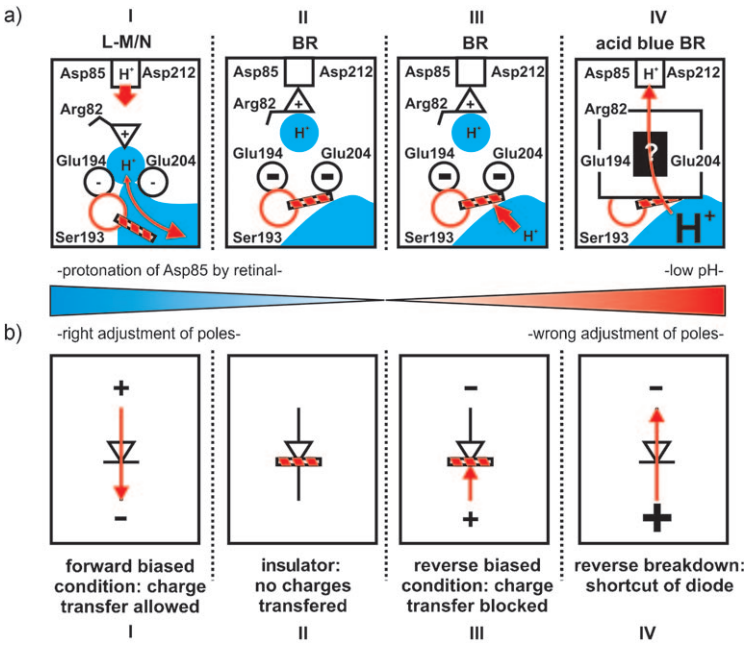

Figure 4. The proton-diode mechanism (a) compared to processes at a diode (b); for details see text.

This mechanism displays features very similar to the directional charge transfer of a diode in electronic devices (Figure 4). Case I is termed "forward biased condition": the proton release in M is equivalent to a correctly applied voltage in a diode, leading to an electric current. Case II and III: the diode is an insulator without voltage (II) or a low counter voltage (reverse bias condition) applied. This situation corresponds to the situation in the bR ground state: The Ser193/Glu204 gate prevents proton transfer between the bulk and the protein interior. In case IV, the reverse bias exceeds the so-called peak inverse voltage; there is a charge flow through the diode in the direction opposite to the one in case I. Complementary, at pH < 2.5, Asp85 becomes protonated from the external bulk in the bR ground state, forming the acid blue form of bR.[28] Acid blue bR absorbs photons but without proton pumping. Because of these striking similarities, we now term this mechanistic feature in bR a "proton diode". The protonated water cluster shields Asp85 from the protein exterior and ensures its deprotonated state in BR. This process ensures high efficiency of the proton-pumping mechanism, as a protonated Asp85 in the bR ground state would result in a waste of absorbed photon energy. The size of this "proton diode" is on the scale of 1.5 nm. Therefore, bR contains a charge-gating device, which is far beyond the state-of-art of miniaturization in nanotechnology.

The protein family of microbial rhodopsins combines a high structural similarity with a large functional diversity. Recently, it was shown that one of its members, proteorhodopsin, plays a major role in the conversion of light to energy in marine bacterial plankton, providing a large part of the marine proteome.[7] Figure 5 shows a comparison of the protein-internal water cavities in crystal structures of bacteriorhodopsin[19] and the microbial rhodopsins sensory rhodopsin (SR) II,[29] xanthorhodopsin,[30] and halorhodopsin.[31] While bulk solvent is blocked by the proton diode at the extracellular protein surface in bR, a direct connection of water molecules up to the Schiff base counterion (Asp75) exists in SR II. In halorhodopsin and xanthorhodopsin, water can penetrate into the protein up to the analogue of Arg82 in bR. Only bacteriorhodopsin contains the two glutamates and

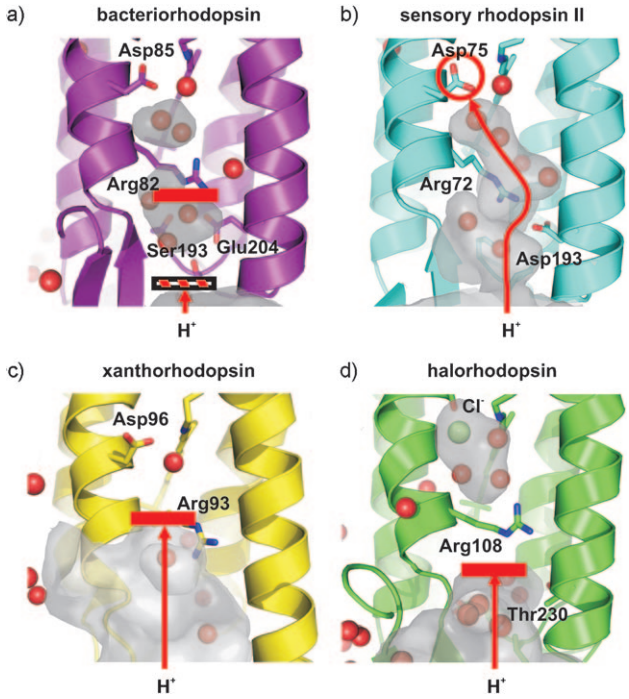

Figure 5. Water-filled cavities of bR-like proteins and their accessibility from the bulk solvent. Protein Connolly surfaces in gray. a) Bacteriorhodopsin (PDB-ID 1C3W,[19] 1.55 Å); b) sensory rhodopsin II (PDB-ID 1H68,[30] 2.1 Å); c) xanthorhodopsin (PDB-ID 3DDL,[31] 1.9 Å); d) halorhodopsin (PDB-ID 1E12,[32] 1.8 Å).

one serine for the proton diode (Figure 4 in the Supporting Information). Consequently, its counterion of the central proton-binding site has a pK_a of 2.5.[28] SR I and II, proteorhodopsin, and xanthorhodopsin exhibit a much higher pK_a of the counter ion (between 5.6 and 7.5),[32–35] indicating an increased sensitivity to external protonation. Sensory rhodopsins are capable of proton pumping when they are not connected to their transducer molecules, but this process is much less efficient than bR.[7] Xanthorhodopsin, a proton pump like bacteriorhodopsin, harbors a carotenoid as a light-harvesting complex[30] and can thus use light in an evolutionarily different way and much more efficiently than bR. Marine organisms expressing proteorhodopsin have an environmental pH of 7.8 to 8.0, so proteorhodopsin does not need to shield its interior against a low external pH.[36] Hence, the proton diode appears to be an evolutionarily optimized modular feature of bR to raise the efficiency of its proton-pumping mechanism and to render it less sensitive to extreme external conditions.

Summing up, directional proton transfer in bR via protein-bound water molecules functions similarly to an electronic diode. Therefore, we refer to the proton-release group as a "proton diode". A downward movement of Arg82 opens a gate to the protein exterior formed by Ser193 and Glu204. Bulk solvent and the internal protonated water cluster merge and perform a directional Grotthuss proton transfer to the external medium. As protein-internal water molecules are found in many structures of membrane proteins and discussed as playing an active part in their proton-transfer mechanism,[3,4] we are convinced that directional proton transfer in other membrane proteins may involve a "proton diode" as well. The protonated water complex, called Eigen cation, is now identified within a membrane protein and is part of the proton-transfer mechanism. The observation of Manfred Eigen in liquid water and ice is now extended to proteins.[37]

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