Wide Dynamic Range Kinetic Studies of Deep Proton Tunneling in Proteins

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Abstract:

Directional proton transport along “wires” that feed biochemical reactions in proteins is poorly understood. Amino acid residues with high pK_a are seldom considered as active transport elements in such wires because of their large classical barrier for proton dissociation. Here we use the light-triggered proton wire of the green fluorescent protein (GFP) to study its ground electronic state proton transport kinetics, revealing a large temperature-dependent kinetic isotope effect. We show that “deep” proton tunneling between hydrogen-bonded oxygen atoms having a typical donor-acceptor distance of 2.7-2.8 Å fully accounts for the rates at all temperatures, including the unexpectedly large value ($2.5 \times 10^9 \text{ s}^{-1}$) found at room temperature. The rate limiting step in GFP is assigned to tunneling of the ionization-resistant serine hydroxyl proton. This suggests how high pK_a residues within a proton wire can act as a “tunnel-diode” to kinetically trap protons and control the direction of proton flow.

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Proton transport (PT) in biological systems has been the subject of many studies, both experimental¹⁻⁶ and theoretical⁷⁻¹². A better understanding of this phenomenon is sought because it underpins so many fundamental life processes¹³⁻¹⁸. One particularly important process involves the delivery of protons to the interior of proteins so that key biochemical reactions can proceed¹⁹⁻²¹. In the case of the oxidative phosphorylation complexes, such as cytochrome oxidase, and in the **photosynthesis complex, cytochrome b₆f**, vectorial proton pumping is also involved. Some of these PT processes are thought to occur by means of “proton wires”, but such wires often contain fluctuating or transient water molecules so that their composition can be difficult to resolve with structural methods. These wires are often modeled using classical methods so that ionization-resistant amino acid residues, such as serine and threonine with their large barriers for proton dissociation, are seldom included as active transport elements. On the other hand, if these residues had significantly faster PT rates, they might be gainfully included in proton wire motifs as active transport elements, as well as structural components that help to stabilize water molecules.

One way to accomplish more rapid PT, when a large barrier is present, is through quantum mechanical tunneling of the proton. This can be described by using a simple model where both the proton vibrational motion and the relative motion between the proton donor (D) and acceptor (A) atoms are factored out and treated at the quantum level, while the remainder of the complex protein dynamics is treated classically using a generalized “environmental” coordinate. In Figure 1, we depict the configuration of the proton and D-A vibrational potentials at the transition state determined by the environmental coordinate. The transition state is reached when thermal fluctuations bring the reactant and product state energies of the quantum sub-space into a condition of degeneracy so that tunneling can occur. The broad, red-shaded, arrow depicts

“deep-tunneling” of the proton through a large barrier between the donor and acceptor oxygen atoms. For typical OH---O hydrogen bonds, this results in the transport of the proton through a distance of $\sim 0.7\text{-}0.8$ Å. However, as the extent of the D-A motion is increased by thermal excitations, shorter tunneling distances become accessible. The associated increase in the tunneling rate is depicted by the heavier red shading on the arrow.

When the donor oxygen has a high pK_a within the protein interior, and the oxygen acceptor is neutrally charged, the barrier along the OH coordinate can remain high throughout the red-shaded region in Fig. 1 so that the overall rate is dominated by the deep tunneling process. As will be seen from the analysis of the experiments presented below, the rate associated with room temperature deep tunneling under this condition is surprisingly fast ($\sim 10^9$ s $^{-1}$). For example, it exceeds by many orders of magnitude the rate that would be found by using an Arrhenius expression to pass over the saddle point at energy E_c in Fig. 1 (see Supplementary Discussion 8 for details). On the other hand, if the donor is more acidic and the barrier is smaller, a more energetically favorable route may involve trajectories that bring the system closer to the saddle point where “small-curvature” tunneling can take place near the top of the barrier.

The deep tunneling channel, or “large curvature tunneling”^{10,22}, dominates the rate when the barrier along the OH coordinate is much larger than the zero-point energy of the OH oscillator. Under these conditions, the proton vibrational levels of the reactant and product states are only weakly mixed and their energy splitting (Δ_0) is much smaller than $k_B T$. Although this condition is often associated with a large kinetic isotope effect (KIE), it is worth pointing out that such effects may not be clearly revealed in more complicated multi-step biological reactions where the measured rates are much slower than the deep tunneling time-scale.

In this work, we have utilized the green fluorescent protein (GFP) as a model system because the chromophore can be used to optically trigger transport on a proton wire. This allows the thermally equilibrated, *ground* electronic state PT to be monitored over an unprecedented dynamic range of time and temperature. The proton wire in GFP includes amino acid components that are found in other biological systems¹⁷ and, importantly, this wire has evolved to include a high pK_a residue (Ser205) as an active transport element. Thus, the detailed experiments presented here provide insight on the structure and function of such wires as well as an opportunity to test various theoretical models that apply to PT in proteins^{8,10,12,22-27}.

As discussed above and depicted in Fig. 1, we analyze the kinetic data using a simplified (but analytic) deep tunneling model²⁷ that factors out the key vibrational motions of the tunneling particle and the donor and acceptor oxygen atoms, while treating the rest of the surroundings using a generalized environmental coordinate. Because there is no electronic state change during the reaction, it is considered to be electronically adiabatic^{12,22}. This allows the rate to be calculated on an absolute scale, without having to estimate the unknown electronic matrix element that appears in the electronically non-adiabatic limit (e.g., when longer range electron transport is also involved). In the approach used here, the reorganization energy of the surroundings, λ_s , is the only unconstrained parameter needed to account for the data and the surprisingly fast proton tunneling rate (~ 400 ps) measured at 295 K¹.

The results indicate that deep tunneling is the rate limiting proton transport event involved in resetting the GFP proton wire for $T \lesssim 295$ K. The importance of room temperature deep proton and hydrogen tunneling in biological systems remains a somewhat controversial topic, especially in the context of enzyme reactions^{5,11,28,29}. Thus, the experiments presented here, which are the first temperature dependent measurements of tunneling in a biological proton

wire and cover a much larger temperature range than previous studies^{2,3,5,9}, can help us to better understand this important process. Because the deep tunneling rate is so exquisitely sensitive to tunnel-distance variations, it has the potential to play an important role in controlling transport along protein wires or in enzymatic reactions^{5,19,20}.

Results

Temperature Dependent Kinetics of the GFP Photocycle

The GFP photocycle begins in the ground state (*A*) and photon absorption by the chromophore forms the excited (*A*^{*}) electronic state. Protons are rapidly (~10 ps) transported³⁰ from *A*^{*} through a hydrogen bonding network to form state *I*^{*} as shown in Fig. 2. The ensuing green fluorescence from *I*^{*} to *I* takes place in about 3 ns. Following this, the protons return to their initial (*A*) state from the *I*-state via a thermally driven proton transfer process, which is the reaction we focus on in this study. The free energy upon going from *I* to *A* is $\langle \Delta G_{IA}^0 \rangle \sim 8$ kJ/mol, based on an analysis of the absorption band temperature dependence (Supplementary Discussion 2).

Figure 3 presents the first experimental data that probes the temperature dependence of the complete GFP photocycle kinetics and, more specifically, the thermally driven proton transport from *I* to *A* (additional data in Supplementary Discussion 3). The data in Fig. 3 are plotted on a logarithmic time axis to show the differential absorbance change over the full photocycle at the 420 nm *A*^{*}-*A* isosbestic point. The isosbestic probe reveals the appearance of the *I*^{*} state on the ~10 ps time scale as well as the *I*^{*} → *I* population transfer. Near room temperature, interference of the *I*^{*} → *I* process prevents detection of *I* → *A* unless a stimulated emission pump-dump-probe protocol is used¹. However, k_{IA} can be measured at lower temperatures or by using D₂O.

The data must be analyzed over the full time domain in order to generate accurate time constants. We note that the $I \rightarrow A$ kinetics are not perfectly exponential and we therefore find an average rate at each temperature (see Supplementary Discussion 1) and return later to the issue of the (very minor) conformational disorder in the D-A equilibrium tunnel distance distribution that accounts for the non-exponential nature of the observed rates. An Arrhenius plot of the $I \rightarrow A$ rates as a function of temperature and isotopic substitution is given in Fig. 4, which also shows the $I^* \rightarrow I$ rates extracted from the fits (green points). It can be seen that there is a major T-dependent kinetic isotope effect (KIE) in the $I \rightarrow A$ rates, amounting to a factor of 40 at 295 K and a factor of 73 at 160 K. The room temperature rates under various conditions are compared in Supplementary Table 3.1. There is excellent agreement with prior work, with the exception of the measurement in D₂O by Kennis et al.¹, where the $I \rightarrow A$ rate was overestimated because of the limited (4 ns) experimental time window.

Deep Tunneling Models

Large temperature dependent KIEs in enzyme reactions have been observed over much narrower temperature ranges (shaded grey in Fig. 4) and slower time scales^{5,31,32} and are often attributed to conformational (classical) distributions of the D-A distance^{4,5,7,33-35}. Other approaches^{12,27,36} factor out and quantize the D-A vibration (~ 100 fs), as well as the much higher frequency (~ 10 fs) vibrational mode of the tunneling particle, which is a common feature in all analytical models (see Supplementary Discussion 4). Computationally based transition state theories have also been applied in the large curvature limit to account for deep tunneling²².

Within the class of “factorized” analytical models^{12,22,27,36}, thermally driven fluctuations of an independent environmental coordinate lead to a transition state where the vibrational eigenstates of the reactant and product state are degenerate. Under this condition, the proton

zero-point vibrational energy levels mix and shift by the tunnel splitting, Δ_0 . The proton vibrational wave function samples tunnel path lengths, $\ell(R)$, that are smaller than the equilibrium tunnel distance, ℓ_0 , when the D-A oscillator coordinate, R , is compressed relative to its equilibrium position, R_0 . This means that the tunnel splitting (and the associated tunneling rate) will depend on the D-A vibrational state. Details concerning the tunnel splitting, $\Delta_0(R)$, can be found in Supplementary Discussion 4 and elsewhere²⁷.

Because of the wide range of temperatures explored in this work, we treat the D-A vibrational motion as quantized^{12,27,36} and consider it in the context of all normal modes of the protein by using an Einstein oscillator approximation. An analysis of the normal modes of GFP in the *I*-state, as determined using hybrid ONIOM(QM:MM) methods³⁷ (see Supplementary Discussion 5), demonstrates that a single D-A quantum oscillator, with a frequency $\bar{\omega}_E/2\pi c \cong 264\text{-}289\text{ cm}^{-1}$ and a reduced mass $\mu_{DA} = 8.5 \pm 0.5\text{ amu}$, effectively describes the temperature dependence of the protein-driven D-A oxygen atom motion in GFP. In addition, there is a mandated Duschinsky rotation (or mixing angle, θ) between the quantized reactant and product normal modes composed of the mass-weighted D-A and tunneling particle vibrations. This angle is determined by the reduced masses of the two oscillators (see Supplementary Discussion 4).

In the following, we assume that a single deep proton tunneling reaction is the rate-limiting event for the overall transport process in GFP (i.e., the other two protons follow in asynchronous but concerted fashion). This assumption is based on the longer tunnel distances and larger reorganization energy that must be invoked for simultaneous (synchronous) multiple proton tunneling. The longer tunnel distance is not compatible with the magnitude and temperature dependence of the measured rates as discussed in Supplementary Discussion 7.

Protein Conformational Distributions

We treat the ensemble of protein conformers independently of the fast (~ 100 fs) H-bonded D-A oscillator motion and note that a typical conformational interconversion time ($\sim \mu\text{s}$ - ms) is slower than the GFP tunnel rate (~ 1 ns). This leads to a conformational distribution of the D-A *equilibrium* positions and the associated tunnel lengths, $P(\ell_0)$, where $\ell_0 = R_0 - 2l_c$ ($l_c = 0.99\text{\AA}$ is the OH covalent bond length). Because of the rigidly locked β -barrel structure of GFP³⁸, only a very narrow distribution of equilibrium tunnel distances, $\sigma_{\ell_0} \sim 0.02\text{\AA}$ centered at $\bar{\ell}_0 = 0.78\text{\AA}$, is needed to account for the slightly non-exponential kinetic decay (see Supplementary Discussion 6).

Global Fits to the Temperature and Isotope Dependent Rates

In Fig. 4, the analytic deep tunneling expression (Supplementary Eq. 4.6) is used to globally fit the measured rates (red curves). We set the reaction free energy to its measured value ($\Delta G_{IA}^0 = -665\text{cm}^{-1}$), and the O-H mode frequency, $\omega_1(H)$, is fixed using its experimental correlation³⁹ with the tunnel distance. Table 1 shows the results using a quasi-harmonic potential surface²⁷. The analysis involves no arbitrary scaling. In contrast, a low-barrier quartic potential generates unacceptable fits to the data (see Supplementary Discussion 6).

Although three parameters (ω_{DA} , $\bar{\ell}_0$, and λ_s) were allowed to vary when fitting the data, two of these parameters ($\bar{\ell}_0$ and ω_{DA}) have strong independent constraints. The value found for $\bar{\ell}_0 = 0.78\text{\AA}$ ($\bar{R}_0 = 2.76\text{\AA}$) is within the error of both the X-ray structure⁴⁰ and the *I*-state density functional theory (DFT) energy minimization³⁷, where $\bar{R}_0 \sim 2.7\text{\AA}$. The D-A frequency ($\omega_{DA} = 273\text{ cm}^{-1}$) falls well within the range found from the independent Einstein oscillator analysis of the GFP normal modes ($\bar{\omega}_E = 264\text{-}289\text{ cm}^{-1}$). The only completely unconstrained parameter is λ_s , which reflects the reorganization energy of the rate limiting charge transport step. The

relatively small value found for $\lambda_s = 1165 \text{ cm}^{-1}$ is consistent with a sub-Å single proton transport distance. (i.e., a larger λ_s would be expected for synchronous concerted tunneling over a longer distance than for a single proton rate-limited asynchronous concerted tunneling event).

Discussion

The ultrafast excited state ($A^* \rightarrow I^*$) PT in GFP has been used as an optical trigger to observe thermally driven ground state proton tunneling ($I \rightarrow A$) over a wide range of time and temperature. We note that the excited state PT rate is much faster than observed for ground state tunneling, presumably due to the different H-bonding arrangement, the vibrational heating from the absorbed photon, and the excited state photo-acid structure⁴¹, which has a weaker chromophore O-H bond and contracted donor-acceptor oxygen distances²⁶. An interesting conjecture for the excited state process involves diffusive proton movement along an alternative wire and out into solution⁴². Although the present analysis does not explicitly address the excited state transport, prior work⁴³ shows that E222 recruits a proton on the same time-scale as the $A^* \rightarrow I^*$ process, consistent with Fig. 2. Importantly, the $I \rightarrow A$ reaction shows a large KIE demonstrating that tunneling, rather than proton diffusion, is involved in the chromophore re-protonation. Moreover, studies of the E222D mutant (see Supplementary Discussion 3) reveal a significant reduction in the $I \rightarrow A$ rate, confirming that proton delivery to the chromophore is dependent on the H-bond linkage between the carboxyl group of residue 222 and Ser205 as depicted in Fig. 2.

Because the simple factorized deep tunneling model fits the absolute magnitude and temperature dependence of k_{IA} so well, we conclude that this is the dominant channel regulating transport on the proton wire at 295 K. The model utilizes a quasi-harmonic potential energy barrier that is surprisingly high at the 0.78 Å equilibrium distance (~50 kcal/mol), but this barrier

is narrowed and significantly reduced upon vibrational compression of the D-A mode. Because deep tunneling rates are primarily sensitive to the tunnel distance, and less sensitive to the details at the top of the barrier²⁷, a potential with a lower equilibrium barrier (i.e., ≥ 25 kcal/mol) cannot be ruled out based on the kinetic data alone. However, we note (Supplementary Discussion 8) that the OH stretch of bulk (i.e. charge-neutral) water with a ~ 2.8 Å distance between hydrogen bonded oxygen atoms displays harmonic progressions (i.e., vibrational bound states) up to at least the 3rd overtone⁴⁴ (~ 40 kcal/mol). This demonstrates that high quasi-harmonic OH potential barriers must exist in such systems. Moreover, as noted in Supplementary Discussion 6, a low barrier quartic potential generates poor fits to the GFP data. **If a purely classical Arrhenius transition is considered, we find that the barrier must drop below ~ 1600 cm⁻¹ $\sim \hbar\omega_1/2$ in order to account for the magnitude of the room temperature $I \rightarrow A$ rate in GFP (see Supplementary Discussion 8) and this is not compatible with the observed KIE.**

Based on the analysis in Supplementary Discussion 7 as well as the relatively small reorganization energy needed to fit the GFP kinetics, we conclude that an individual proton tunneling step is rate limiting for the $I \rightarrow A$ reaction. Inspection of the GFP proton wire indicates that the ionization-resistant hydroxyl of S205 (pKa ~ 13), which is H-bonded to a neutral water molecule, has the high quasi-harmonic barrier that harbors the rate-limiting proton (i.e., the E222 proton is acidic and more easily dissociated, while the water proton is ionizable and H-bonded to the negatively charged oxygen of Tyr66). This assignment also helps explain the faster excited state $A^* \rightarrow I^*$ rate compared to the ground state. In the A^* state, the S205 proton engages in a stronger H-bond to the anionic E222 carboxylate, which should reduce its barrier and transport distance.

On the other hand, mutant studies involving S205V have suggested that E222 might be a rate-limiting donor⁴⁵. However, due to structural rearrangements, an analogous hydroxyl residue (T203) takes the place of S205 in the proton wire of this mutant^{45,46}. Thus, deep tunneling that is rate-limited by the hydroxyl proton of T203 can explain the nearly identical room temperature rates found for the S205V mutant and the wild type. We note that a similar rate invariant mutation effect has been observed in the photosynthetic bacterial reaction center, upon threonine substitution for serine at the terminal site of its proton wire, where protons are delivered to the quinone receptor⁴⁷.

From a broader perspective, these observations demonstrate that ionization-resistant hydroxyl groups, such as serine and threonine (or other residues with a locally high pK_a ^{48,49}) can be H-bonded within a proton wire and still actively participate via deep proton tunneling. Well-aligned proton wires that are composed solely of water molecules are known to be extremely efficient charge carriers that can transport an excess proton on a sub-ps timescale²⁵; however, these water structures can be unstable, especially when long distances need to be traversed⁵⁰. Thus, the high pK_a residues (such as Ser and Thr) can play an important role in proton wires by stabilizing shorter segments of water molecules and by helping to control the proton flow.

Attenuation of the proton current, due to slower transport at the tunneling sites may help in coupling to biochemical reactions where a regulated proton flux with added wire stability is desirable. Directionality can be kinetically controlled because of the exquisite sensitivity of the rate to the tunnel distance. As depicted in Fig. 5, a slightly shorter tunnel distance for the hydroxyl proton as it points downstream (ℓ_d), in the desired direction of proton flow, compared to when it points upstream (ℓ_u) will lead to very slow back transport rates and a “tunnel diode” effect.

Although participation of amino acid hydroxyl residues as active elements in the PT chain was an initial suggestion for proton wires^{18,51}, high-level theoretical simulations that include such residues are rare. It has been proposed that the hydroxyl residue must be protonated to -OH_2^+ in order to make transport to the adjoining neutral water possible¹⁷, but such a protonation step has very low probability. Generally, when a classical approach is used, large barriers associated with high pK_a residues in the protein interior^{48,49} will lead to such slow rates that these residues must be excluded. In contrast, the experimental results presented here demonstrate that such residues can play an active role in proton wires via deep tunneling and suggest a natural way for the protein to control the direction of proton flow.

Figure 5 shows amino acid residues with high pK_a as active elements in a hypothetical proton wire. The downstream proton current is attenuated by the $\sim\text{ns}$ deep tunneling rate, k_{ℓ_d} , which is much slower than the $\sim\text{ps}$ time-scale proton hopping rate, k_{Hop} , found for transport within the water segments²⁵. A shorter segment of the wire is shown in panels b and c of Fig. 5, depicting how the rate-limiting tunneling step moves a hydronium ion from an “upstream” to a “downstream” position relative to the serine hydroxyl group. This occurs via a concerted asynchronous tunneling process with the concerted formation (straight arrow labeled “con”) of a new H-bond that points upstream. The upstream hydronium ion in panel b donates its proton in a concerted asynchronous step because of its positive charge and its lower barrier compared to the rate-limiting tunnel barrier between serine and the downstream neutral water molecule. **Thus, the ionization-resistant serine hydroxyl will act as a “tunnel diode” so that the newly created downstream H_3O^+ will continue to hop preferentially downstream along the water segment.** The fact that $k_{Hop} \gg k_{\ell_u}$ means that the proton flow will continue away from the hydroxyl tunnel junction and further down the water segment via sub-ps hopping rather than waiting for the Ser-

OH proton to tunnel back upstream through the distance ℓ_u (Fig. 5c). When the upstream tunnel distance, ℓ_u , is larger than ℓ_d , the timescale for tunneling back upstream can, in principle, be made arbitrarily slow, so that the positive charge can be kinetically trapped and maintained downstream under non-equilibrium conditions. As the proton moves further downstream along the water segment, the Ser-OH may also reorient and re-establish its original H-bond configuration with the adjacent downstream neutral water (note that, when $\ell_d < \ell_u$, the shorter downstream hydrogen bond is stronger and energetically more favorable). This reorientation will help to trap the positive charge downstream as well as help to initiate a reset of the overall hydrogen bond configuration in the wire so that the next incoming proton can be accommodated. The reorientation of the remaining confined water molecules may also benefit from the energy dissipated by the (exothermic) reaction that is being fed by the proton flux.

If the ionization-resistant hydroxyl residue lies near the beginning of a water wire, the tunneling process could also act as an effective trap for protons that are recruited into the channel. Thus, upon tunneling past the initial hydroxyl residue, the proton can hop along a water segment and work its way down the wire to the final acceptor site where it is consumed by a coupled biochemical reaction. The respiratory complexes¹⁹ contain numerous serine and threonine sites that are associated with proton wires, but many of the detailed pathways remain ambiguous because of insufficient X-ray structure resolution⁵². On the other hand, the presence of serine at the terminal position of the proton wire in the bacterial photosynthetic reaction center is well-established²⁰; moreover, serine and/or threonine have been identified in a number of other biological proton wires^{53,54}, indicating that the rate-limiting deep tunneling reaction in GFP is relevant to a broader range of systems.

We conclude that the ground state proton wire of GFP is rate-limited by a reaction that involves the unexpectedly fast deep tunneling of a single proton associated with the ionization-resistant S205 residue. This demonstrates that hydroxyl residues with a large pK_a can actively participate in proton wires in proteins via deep tunneling and suggests more generally how high pK_a residues in the protein interior can provide stabilization, directionality, and regulation of proton flow.

Methods

Experimental Setup and Sample Preparation. GFPuv was prepared as described in⁵⁵ and dissolved in a 20 mM H₂O Tris_HCl buffer (pH 8.0) with 75 mM KCl. For deuterated samples, a buffer exchange was conducted by using a highly concentrated GFP sample and diluting it in 20 mM Tris-DCl buffer at pD 8.0. This sample was loaded into a centrifuge and re-concentrated and the complete procedure was repeated three times. The sample was then sealed and left at room temperature for 48 hours to ensure efficient H-D exchange of the protein. All samples were in 50% glycerol, where glycerol-OD₃ (hydroxyl protons are exchanged for deuterium) was used for the deuterated samples.

The temporal kinetic traces were measured using a two color pump-probe instrument that has been described in detail elsewhere⁵⁶. The instrument involves two Ti: sapphire regenerative amplifiers, both operating at 190 kHz, which are seeded by two synchronized Ti:sapphire oscillators: a master (Mira-P, 76 MHz, 3 ps (FWHM)) and a slave (Mira-F, 76 MHz, 100 fs (FWHM)). The output of the femtosecond regenerative amplifier (Coherent REGA9000) was focused in a 500 μ m BBO crystal to generate the second harmonic at 403 nm, which was used to pump the sample. The amplified picosecond pulses from a tunable picosecond regenerative amplifier were doubled using a 2 mm BBO crystal. This beam was tuned to 420 nm. The two

beams were then collinearly combined and focused in the sample using an achromatic lens with a 2 inch focal length. The time delay between the pump and probe pulses was controlled electronically using a phase shifter in the synchrolock system (continuous scan from 0 to 16 ns) along with the ps regenerative amplifier timing delay electronics that allow extending the scan window up to the inverse of the repetition rate ($\sim 5 \mu\text{s}$) by discrete steps of 13 ns. The timing jitter between the pump and probe pulses is ~ 800 fs. The polarization of the pump and the probe beam are set to the magic angle ($\sim 54.7^\circ$) to eliminate rotational relaxation artifacts. The sample was loaded in a 1 mm pathlength PMMA cell. The cell was then mounted in a cryostat equipped with a digital temperature controller. After the sample the pump beam was blocked by a long pass filter and the change in transmission of the probe was detected by a photodiode connected to a lock-in amplifier, where the pump beam was modulated at 2kHz using a mechanical chopper. Fast optical scanning across the sample was performed with an x-y galvo scanner. To preserve the integrity of the sample and minimize the permanent photo-conversion we used a fresh sample for each temperature. Fast scanning of the sample, timing the delay between the pump and the probe (from fs to μs) and data acquisition were controlled using a Labview program.

Einstein Oscillator Approximation to Donor-Acceptor Motion. As discussed in detail in Supplementary Discussion 5, the temperature dependent D-A vibrational motion, arising from the cumulative effects of the protein normal modes, can be characterized by the relative mean square displacement, $\sigma_{DA}^2(T)$. This quantity can be well-approximated by invoking an “average” (Einstein) oscillator with reduced mass, μ_{DA} , and frequency, $\bar{\omega}_E = \sum \omega_\alpha \sigma_\alpha^2 / \sum \sigma_\alpha^2$, which is defined by the power spectrum of normal modes driving the relative distance between the donor and acceptor atoms. The quantity σ_α^2 is the mean square Cartesian displacement along the unit vector between the donor and acceptor that is derived from each normal mode (α) of the protein-

chromophore system. The normal modes of GFP in the *I*-state, as determined using hybrid ONIOM(QM:MM) methods³⁷, allow us to find the mode-specific value, σ_{α}^2 , for each pair of oxygen atoms shown in red in Fig. 2. The associated average frequencies and reduced masses can then be calculated and the result is $\bar{\omega}_E/2\pi c \cong 264\text{-}289\text{ cm}^{-1}$ and $\mu_{DA} = 8.5 \pm 0.5\text{ amu}$.

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Figure Captions:

Figure 1. Deep tunneling at the environmental transition state. Two potential channels of proton transfer are shown. The red arrow represents deep tunneling along the proton coordinate through a high barrier. The square of the symmetric ground state two-dimensional proton vibrational wavefunction is indicated by the colored ovals. Shorter tunnel distances become more accessible at higher temperature as the D-A probability distribution spreads along the low frequency donor-acceptor coordinate. The green dashed arrow is a classical pathway showing the energy, E_c , needed to compress the D-A bond and reach the saddle point. Using a typical ionization resistant O-H---O configuration and a standard attempt frequency leads to a classical room temperature Arrhenius rate that is ~ 6 orders of magnitude smaller than the deep tunneling rate (see Supplementary Discussion 8 for more detail).

Figure 2. The photocycle and chemical structure of GFP. a) Upon excitation of the absorption band associated with the $A - A^*$ transition using 398 nm light, the chromophore undergoes picosecond excited state proton transfer to form an intermediate state, I^* , with an anionic chromophore (this is a bi-exponential ultrafast process denoted here simply as $k_{A^*I^*}$). After fluorescence emission to the anionic ground state, I , the chromophore is reprotonated via ground state proton transfer with a rate denoted as k_{IA} . As shown in Supplementary Discussion 2, the free-energy gap, ΔG_{IA}^0 , between I and A is -8 kJ/mol (or -665 cm⁻¹) and predominantly enthalpic. b) Chemical structure of the A state with a protonated chromophore. c) Chemical structure of the I state with an anionic chromophore.

Figure 3. Transient absorption measurements as a function of temperature for GFP in a) H₂O and b) D₂O. A 150 fs pump pulse at 405 nm was used to excite the sample and a 3 ps pulse tuned to 420 nm, the isosbestic point¹ between A and A^* , was used to probe the sample. The red lines are fits to the data over the full photocycle using exponentials to account for the two phases of decay from $A^* \rightarrow I^*$ the radiative decay from $I^* \rightarrow I$, and the slightly non-exponential proton transport from $I \rightarrow A$. A Gaussian instrument function (~ 3 ps) was also included in the fits and the system time-zero is denoted as t_0 . Further details are given in Supplementary Discussion 1.

Figure 4. Arrhenius plot of the ground state ($I \rightarrow A$) proton transfer rates in GFP. The associated error bars are smaller than the data points and are not shown. The typical temperature range for studies of biological hydrogen and proton transport is shaded in grey. The rate for the H₂O samples (in 50% glycerol buffer) increases at higher temperature and becomes inseparable from the 3 ns $I^* \rightarrow I$ population transfer due to fluorescence decay (green points). The apparent decrease in the $I^* \rightarrow I$ rates at higher temperature in H₂O is due to the underlying oppositely signed $I \rightarrow A$ process. A measurement at room temperature in pure buffer (0% glycerol) using the pump-dump-probe method¹ is shown with the open diamond, and has been scaled up by a factor of 1.4 to correct for solvent differences (see Supplementary Discussion 3). The experimental KIEs of 73 (160 K) and 40 (295 K), are depicted by the blue dashed lines. The global fits to the kinetic data, using the model described in Supplementary Discussion 4, are shown as the red lines. The fitting parameters are listed in Table 1.

Figure 5. Hypothetical proton wire composed of water molecules and uncharged amino acid residues with high local pK_a. (a) The PT between water molecules occurs via $\sim$ ps “hopping”, k_{Hop} , so that the downstream deep proton tunneling from the high pK_a hydroxyl group, k_{ℓ_d} , becomes rate-limiting (straight arrow). The [H₂O]_n indicates a group of n water molecules. (b) A shorter segment of the wire near the serine shows an upstream hydronium ion poised to undergo an asynchronous concerted transport step that is rate-limited by k_{ℓ_d} . When the serine proton tunnels to the downstream neutral water, the hydronium proton undergoes a concerted transfer to serine, denoted by a straight arrow labeled “con”. (c) The wire is shown just after formation of the downstream hydronium with serine H-bonded to an upstream neutral water. The tunneling rate back upstream is denoted by k_{ℓ_u} . Because $k_{Hop} \gg k_{\ell_u}$, the charge can

be kinetically trapped downstream. If $\ell_u > \ell_d$, trapping holds for very long time-scales and the downstream serine hydrogen bond is stronger and energetically more favorable. Thus, it should reorient to point downstream, further trapping the charge, even against a proton gradient. The system resembles a “tunnel diode”. A downstream proton sink (e.g., a biochemical reaction) helps to maintain proton flow.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature

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Table 1. Parameters for ground state proton transfer kinetics in GFP

Unconstrained	Value	Description
λ_s	$1165 \pm 20 \text{ cm}^{-1}$	Reorganization energy
Constrained*		
$\omega_{DA}/2\pi c$	$273 \pm 7 \text{ cm}^{-1}$	D-A mode frequency (fit)
$\bar{\ell}_0$	$0.78 \text{ \AA} (\sigma_{\ell_0} = 0.02 \text{ \AA})$	Equilibrium tunnel distance
Fixed†		
$2b_0/d_0$	<0.2	Width of inverted parabola
ΔG^0	-665 cm^{-1}	Free energy of $I \rightarrow A$ reaction
$\omega_1(H)/2\pi c$	3330 cm^{-1}	OH mode frequency
μ_{DA}	$8.5 \pm 0.5 \text{ amu}$	Reduced mass of D-A mode
θ_H	$19.2^\circ \pm 0.5^\circ$	Duschinsky rotation for H
θ_D	$26.5^\circ \pm 0.7^\circ$	Duschinsky rotation for D
χ_{red}^2	8.0×10^{-3}	Reduced chi-squared

* $\bar{\ell}_0$ is constrained by X-ray data and I-state ONIOM minimization and σ_{ℓ_0} is the width of $\bar{\ell}_0$ distribution.

* $\omega_{DA} \cong \bar{\omega}_E$, is constrained to 264-289 cm^{-1} as calculated and shown in [Supplementary Fig. 5.1](#).

† The O-H frequency of the proton, $\omega_1(H)$, was correlated³⁹ to the O---O distance and fixed by the value of $\bar{\ell}_0$. The parameter b_0 locates the attachment of a continuous inverted parabola at the top of the barrier²⁷. The effect is negligible on the other fitting parameters if $2b_0/d_0 < 0.25$ ($d_0 = \sqrt{\mu_L} \ell_0$). Measurements of $\Delta G^0 = \Delta G_{IA}^0$ are presented in [Supplementary Discussion 2](#). The effective mass for the D-A oxygen motion is $\mu_{DA} \cong 8.5 \pm 0.5 \text{ amu}$ (see [Supplementary Discussion 5](#)). The rotation angles are given by $\theta_L \cong \sqrt{\mu_L/\mu_{DA}}$ and more detail can be found elsewhere²⁷. The uncertainty in μ_{DA} generates uncertainty in the other parameters as noted in the Table (see [Supplementary Discussion 6](#) for more details).

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