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Photoinduced proton transfer in differently structured water. An EPR approach to solving a classic problem

Antonio Barbon [1*], Anton Savitsky[2], Igor Grigoriev[3], Vladimir Reznikov[3], Igor Kirilyuk[3], Sofya Lushchekina[4], Ilia Moroz[5], Tamar Eliash[5], Noga Friedman[5], Mordehai Sheves[5], Raanan Carmieli[6], Lev Weiner[6,7*]

Department of Chemistry, University of Padova, Italy[1], Max Planck Institute for Chemical Energy Conversion, Mülheim an der Ruhr, Germany[2], Novosibirsk Institute of Organic Chemistry, Novosibirsk, Russia[3], Department of Biomolecular Sciences[4], Department of Molecular Chemistry and Materials Science[5], Department of Chemical Research Support[6], Department of Brain Research[7], Weizmann Institute of Science, Rehovot, Israel

*Authors to whom correspondence should be addressed:
antonio.barbon@unipd.it; leweiner1950@gmail.com

Abstract

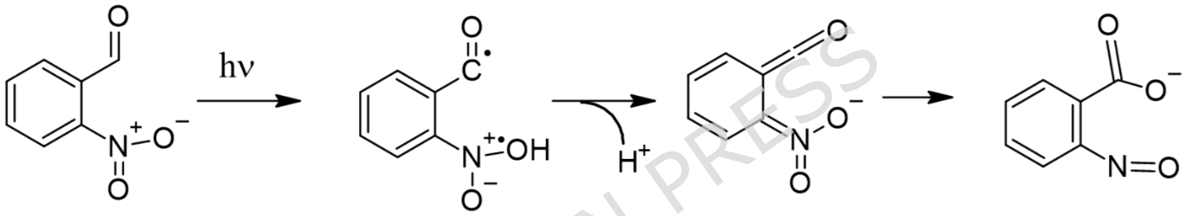
The mechanism of proton transfer (PT), and how it is affected by water structure, is a fundamental issue in numerous chemical and biological processes. Formulated more than 200 years ago, a possible model for PT in aqueous media was proposed by Grotthuss, which continues to be actively studied and debated. In this study, we present an electron paramagnetic resonance approach to investigate PT in aqueous solutions. Our approach employs pH-sensitive stable nitroxyl radicals and makes use of photolysis of 2-nitrobenzaldehyde to generate protons in the sub-nanosecond timescale. The proposed approach was used to study the impact of classical chaotropic compounds on PT as studied in various aqueous solutions, i.e. 8 M urea, 6 M guanidine hydrochloride (Gdn·HCl), and potassium chloride (KCl). Our findings reveal significant impacts on PT rates. For instance, in 6 M Gdn·HCl, PT occurred 40-fold slower than in water. The method's sensitivity to water structure is demonstrated, highlighting its potential for monitoring the kinetics of PT in ice and in proteins.

Introduction

Proton transfer (PT) is a fundamental step in numerous processes in chemical and biological systems [1-4]. A crucial physicochemical issue is understanding of the mechanism of movement of protons through water. Over two centuries ago, von Grotthuss proposed a mechanism for water's electrolytic decomposition [5]. He proposed that proton conduction involves the collective shuttling of protons along water wires associated to hydrogen-bonded water molecules. Many eminent scientists have addressed this issue, in which interest has not faded [6-8]. A brief review in which the Grotthuss mechanism was addressed [9], has been quoted > 4000 times (Scopus database), and with > 240 citations only in 2025 alone

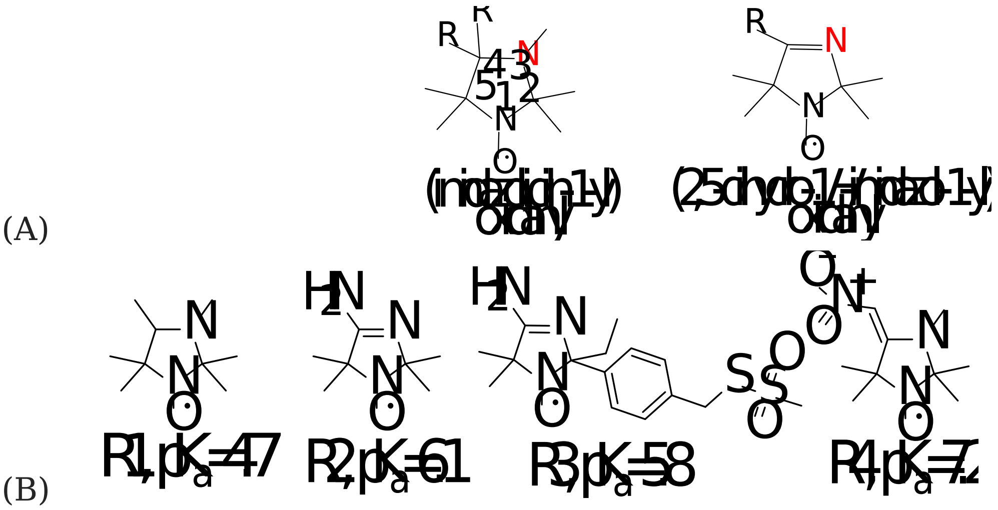
(Web of Science), underscoring its enduring relevance. Despite this intense interest, the PT mechanism remains an open issue. In our opinion, this is due to the lack of reliable experimental methods for quantitatively investigating both the process itself, and how water structure affects it. In this study, we present a novel approach for studying PT, applicable to a variety of systems because of the selectivity of the technique. Here we propose generating protons in aqueous solution through photolysis of 2-nitrobenzaldehyde, 2-NBA [10-12] (see Scheme 1), and using stable nitroxyl radicals of the imidazoline and imidazolidine families (see Scheme 2) as proton acceptors and probes at the same time [13,14]. Therefore, we monitored pH variations over time by electron paramagnetic resonance (EPR) in various aqueous systems containing molecules capable of altering the typical hydrogen-bond water network.

Photolysis of 2-NBA is an effective method for production of protons in aqueous solution. It has been successfully applied to generate fast pH changes in solution and in certain enzymatic systems [10-12] on time scales shorter than picoseconds.



Scheme 1: Photoactivation of 2-NBA resulting in proton generation.

More than 40 years ago it was shown that the EPR spectra of imidazolines (imidazol-1-yl) and imidazolidines (imidazolidine-1-yl) stable nitroxyl radicals (Scheme 2) are pH-sensitive [13,14], and they are currently widely used to measure pH values in various biological and chemical systems [13-19]. Indeed, protonation of the nitrogen atom at position 3 is accompanied by a change in EPR parameters such as the *g*-factor and the nitrogen hyperfine splitting of the nitroxide group[13-19]. This, in turn, permits monitoring of the kinetics of protonation, the PT rate, which is related to the diffusion of protons and the reaction with the probe, while the pH is instantaneously changed due to the photoreaction in Scheme 1. The purpose of this study is to experimentally verify a new approach for the measurement of proton transfer in aqueous solutions using EPR. Since this process is governed by the structure of water, it is important to test this method under sets of controlled conditions like for example in water added with different solutes able to substantially modify the hydrogen-bond structure of water (see further). The advantage of the technique is the selectivity of EPR in the detection of the protonation state of the paramagnetic probe.



Scheme 2. (A) Generic formulae of pH sensitive stable nitroxyl radical's derivatives of imidazoline and imidazolidine (B) Examples of members of these two molecular classes employed in the present study.

Results

Figure 1 shows the EPR spectra of the radical, R1 (see Scheme 2), at various EPR working microwave frequencies with nearly equimolar protonated (RH^+) and non-protonated (R) forms ($pK_a \sim pH$). Both forms are characterized by a classical three-lines spectrum due to the ($N—O^{\bullet}$) interaction of the nitrogen (I=1) with the unpaired electron, delocalized between the two atoms; the two radicals have different *g*-factor and hyperfine interaction (a_N). Note that protonation is a reversible process, i.e., for each value of pH, unprotonated and protonated forms of the radical exist in equilibrium. It is important also to note that these states for the presented radicals (Scheme 2B) are in slow exchange (on the EPR time scale). Due to the different values, of *g* and $a_{N,}$ all three components of the triplet EPR spectrum of the two forms, RH^+ and R, are well distinguished at W-band (140 GHz). At X-band frequencies (9.5 GHz ca) only the high-field components are separated. Of course, when using spectrometers with higher microwaves frequencies, this difference is more pronounced (Figure 1). Note that a theoretical consideration of the effect of protonation on the parameters of the EPR spectra of these radicals (see Scheme 2) well explains the experimentally observed effects [19-21].

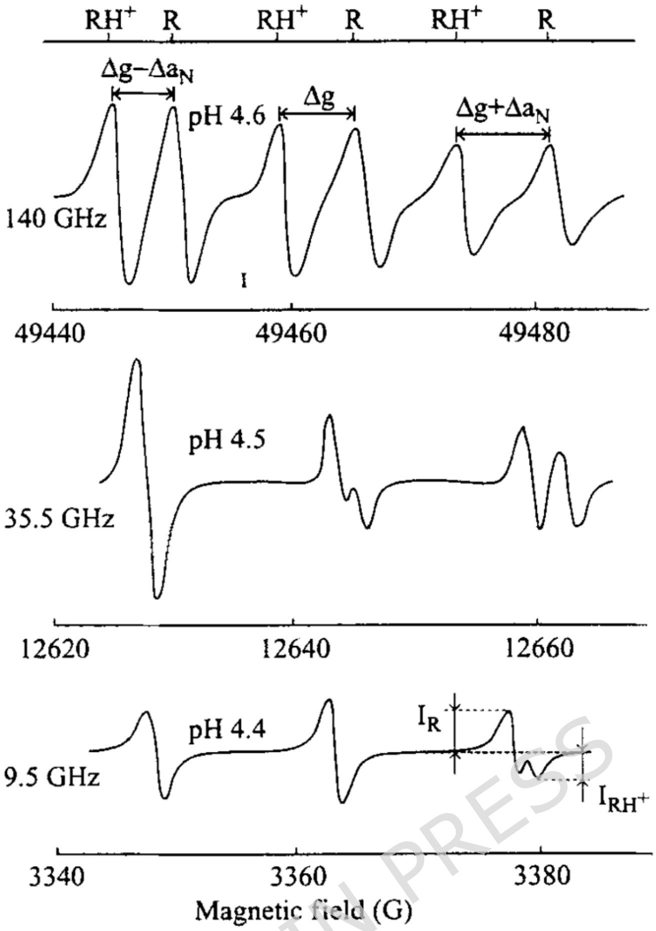


Figure 1. EPR spectra of R1 (at a pH value near its pK$_a$), at different magnetic fields (different microwaves frequencies) in aqueous solutions (from ref. [17] with permission).

Figure 2A shows the change in the EPR spectrum of R2 as function of time during continuous 2-NBA photolysis of a solution with initial pH slightly above the pK$_a$ value. With time the pH value of the solution decreases, and the intensity of the protonated form rises. For comparison, the pH-dependence of the EPR spectrum of R2 is shown (Figure 2B).

The observed time-dependence of the EPR spectrum provides the experimental basis for the proposed method of monitoring PT in time.

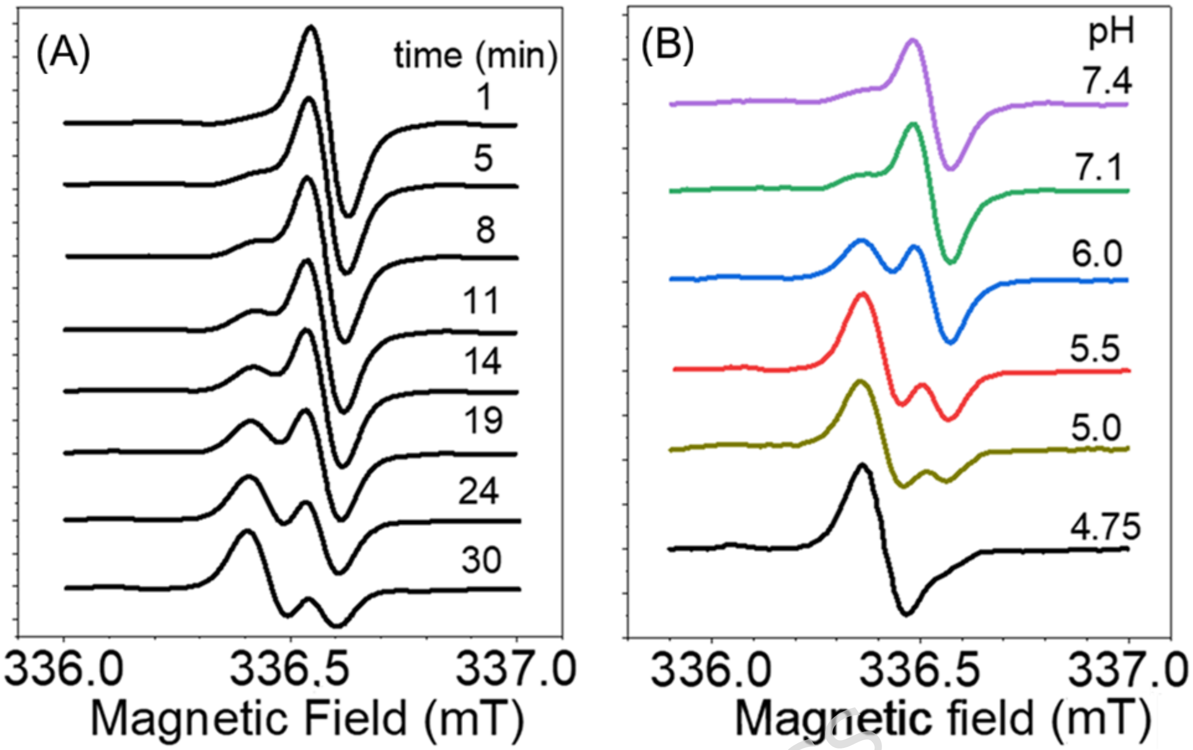


Figure 2: X-band EPR spectra at room temperature of radical R2 (c=50 μM). (A) under continuous irradiation with a Xe lamp of a solution with initial pH 7.5 and in the presence of 2-NBA (c=2 mM), (B) in solution at different pH values. Microwave power was 10 mW, modulation amplitude 0.8 G at 100 kHz,

There is a considerable amount of data in the literature on the effects of salts [22-25] and denaturing agents, such as urea and guanidine hydrochloride (Gdn·HCl), on water structure [26-31]. We used the pH-sensitive nitroxides to monitor the proton diffusion in water solutions containing substances able to perturb the hydrogen-bond network as reported in the literature [26-31].

Figure 3 compares the rates of change in the EPR spectra of R2 in pure water and in aqueous 6 M Gdn·HCl, during the photolysis of 2-NBA[1].

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[1] Note: The pKa values of pH sensitive radicals in the presence of 6 M Gdn·HCl and 8 M urea may differ from the pKa values obtained in water. We determined the pKa values of R2 and R4 under these conditions in a separate experiment (See Suppl. Mat.). It turned out that urea does not affect the pKa value, while in 6 M Gdn·HCl, a shift of pKa of R2 and R4 toward the alkaline side was observed, i.e. from 6.1 to 6.4 (R2) and from 7.2 to 7.4 (R4).

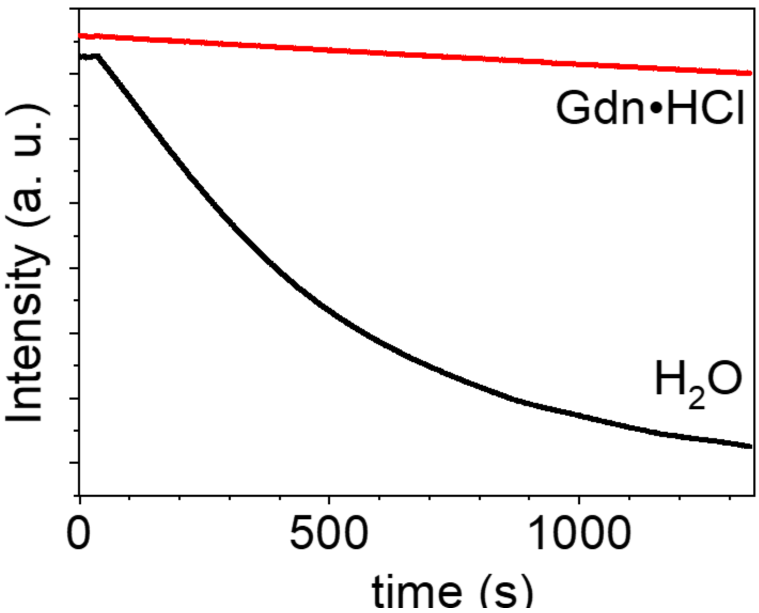

Figure 3: Kinetics of protonation of R2 at pH 7.5 upon continuous irradiation of the solution containing 2-NBA, in the water and in the presence of Gdn·HCl, 6 M measured from the EPR intensity of the non-protonated form at X-band. Concentrations of reagents and experimental conditions like in Figure2.

Table 1 summarizes the pseudo-first order data for PT rates for R2 during 2-NBA photolysis in pure water (Suppl. Info.), and in the presence of KCl, urea and Gdn·HCl obtained under the same irradiation conditions. The rate constants in the table were calculated from experimental kinetics (at least 3 measurements). The error did not exceed 10-15%. The data was fitted as a first-order reaction using Origin software. The significant differences in the PT rates observed support the idea by Grotthus that PT is sustained by hydrogen-bond networks, therefore, addition of solutes which can modify water structure can influence PT rates.

Solution	**Concentration of solute (M)**	***k*** **(s^{-1})**
H_2O	0	$2.3x10^{-3}$
H_2O/Urea	8	$1.04x10^{-3}$
H_2O/KCl	1	$2.7x10^{-4}$
H_2O/KCl	3	$1.9x10^{-4}$
H_2O/Gdn·HCl	6	$5.7x10^{-5}$

Table 1 Variation of the PT reaction after photolysis of 2-NBA obtained under continuous light irradiation of the samples. Experimental conditions: R2 [50 μM], 2-NBA [2 mM]. Starting pH value=7.5. Light power was the same during all experiments.

It is important to point out that the rate of generating free protons in solution during 2-NBA photolysis in solute-free water was the same, within experimental errors, as in the presence of KCl and of the two denaturants, under the experimental conditions used to obtain the data shown in Table 1 (see SM)[2].

The network of hydrogen bonds in 8 M urea is almost identical to that in pure water [26,27]. In contrast, in 6 M Gdn·HCl the network seems to be perturbed [28,29] although for this issue it is still controversial [30,31]. It is thus tempting to ascribe the significant difference between the PT rates observed in 8 M urea and in 6 M Gdn·HCl to the difference in water structure. However, to draw such a conclusion will require a more detailed study, considering also the possible effect of the counter ion, Cl^-, in the case of Gdn·HCl, on the structure of the water [23,24] which was beyond the scope of the present study.

Another important methodological issue that we wanted to solve in this study is testing the possibility of studying PT under conditions in which the free diffusion of particles in a solution is significantly hindered or practically impossible. To this end, we made use of the fact that photoisomerization of 2-NBA to *o*-nitrobenzoic acid is known to occur in liquids and in ice with approximate the same efficacy [11]. We used this property of 2-NBA to test the proposed method in a water-glycerol mixture at 160 K in the “classical glass “[32,33]. Figure 4 shows the time-dependent change in the EPR spectrum of the radical R2 under these experimental conditions. The question of the mechanisms of PT in ice is also extremely important and is the subject of numerous theoretical and experimental studies [11,34-36].

Note that the Grotthuss model is also considered in this case which involves a proton-hopping relay along the hydrogen-bonded water chain of the ice network [34-36]. The observation of protonation in this rigid glassy matrix at 160 K is intriguing and suggests that the classical diffusion-collision mechanism is severely restricted. The exact mechanism, which could potentially involve quantum tunneling [37-39] remains to be elucidated and warrants further investigation, for instance, through kinetic isotope effect studies.

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[2] To discuss on the observed values of the PT rate beside the influence of the water structure, it is necessary to take into account the possible change in the viscosity of the solutions. As follows from the EPR spectra of the R2 and R4 radicals in solutions in the presence of Gdn-HCl and urea, the micro viscosity did not change. At the same time, the macro viscosity in 6 M Gdn.HCl and 8 M urea increased by only 1.6 times (K. Kawahara, C. Tanford, Viscosity and Density of Aqueous Solutions of Urea and Guanidine Hydrochloride. *J. Biol. Chem*. 241,3228-3232 (1966)

Of great fundamental and applied interest is the issue of PT in biological systems, including proteins, membranes, and supramolecular complexes, such as natural and artificial photosynthetic systems [40-43]. In all such systems water is an important component, but PT may also be occurring through structural elements of the macromolecules themselves.

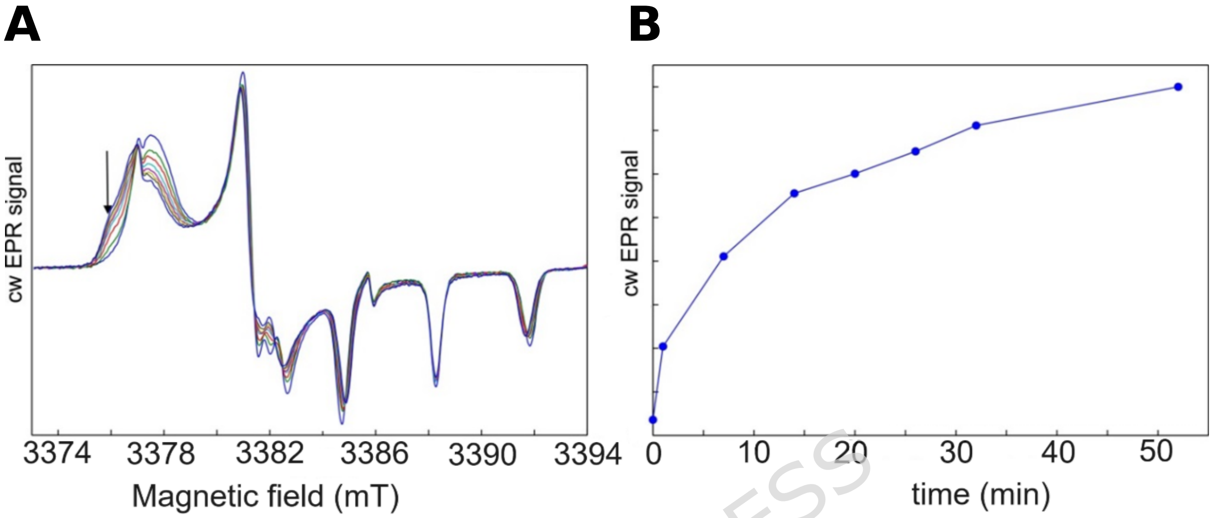

Figure 4 **A)** W-band EPR spectra of a solution of the radical R2 (50 mM) in a water-glycerol mixture 1:1 at 160 K in the presence of 1 mM 2-NBA under irradiation at 355 nm (0.2 mJ/shot and 10 shots/s). The arrow indicates the direction of the evolution of the signal; **B)** the intensity of the EPR spectrum at the field position indicated by the arrow as a function of time.

To test our method on a biological system we utilized protein, bacteriorhodopsin (BR), which has been studied extensively by EPR, making use of site-specific spin labeling [44-46]. The pH-sensitive nitroxyl radical, R3, was introduced by thiol-disulfide exchange at position 163, at which the amino acid present in the native protein, methionine [47] had been replaced by a cys residue by site-directed mutagenesis [44-46]. The EPR spectra of this conjugate is shown in Figure 5A. Figure 5B shows the rate of protonation of the spin labeled protein as a result of photolysis of 2-NBA.

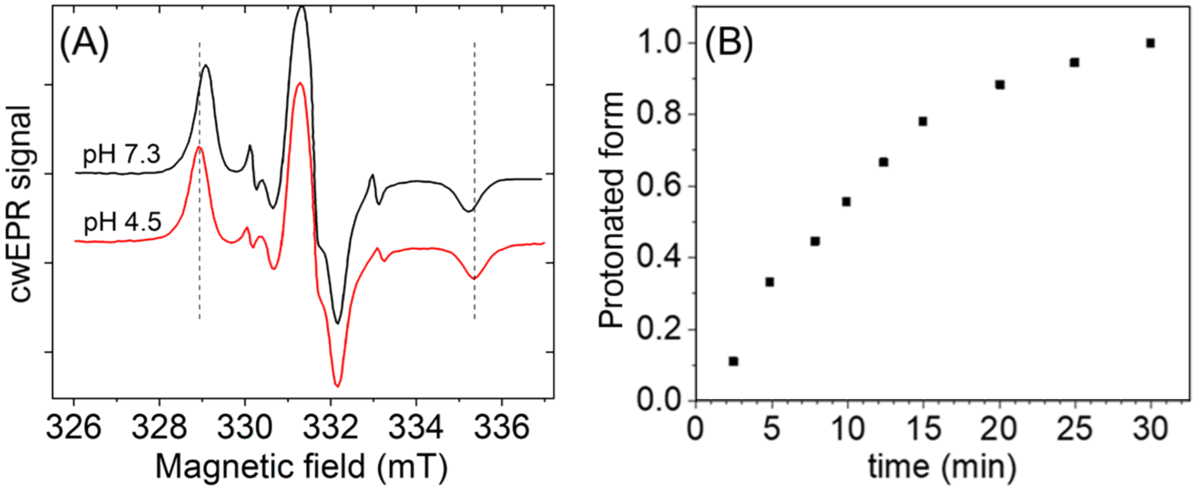

Figure 5 (A**)** X-band CW-EPR spectra of the spin labeled BR,10 mM (black trace, pH=7.3) and of the protonated (red trace, pH 4.5) in water at room temperature (B) the rate of protonation of the spin label (at fixed position in magnetic field 329.1 mT) as a result of photolysis of 2-NBA, 1 mM., Experimental conditions: room temperature, modulation amplitude 1 G, microwave power 20 mW.

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We performed MD simulations of the solvated and membrane-bound spin-labeled BR (the simulation system is shown in Figure 6A) and of a short spin-labelled model peptide in a water solution

Figure 6 shows MD simulation of availability of water to spin labeled mutant of BR and water-soluble spin labeled peptide

The radial distribution functions (RDFs) of water oxygen atoms around the nitroxyl oxygen atoms of the spin labels (Figure 6B) are very similar for both systems. The first peaks of the RDF have the same height and position, which suggests that the water accessibility of the spin-labeled Met163, situated at the protein-membrane-water interface, is comparable to that in a bulk solution. The slightly reduced amplitude of the second peak, corresponding to the second solvation shell, reflects the mild shielding effect of the lipid environment, whereas the first solvation shell remains largely unaffected.

(A) (B)

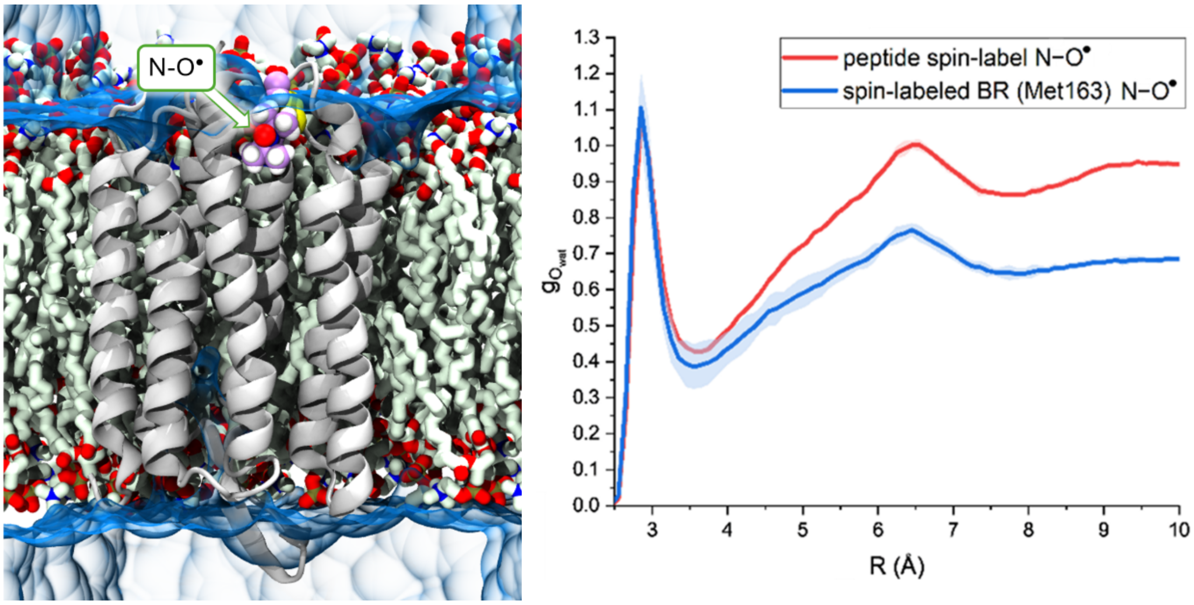

Figure 6. (A) model system of membrane-embedded and solvated bacteriorhodopsin (BR) with the spin label attached at position 163 (shown as VdW spheres, carbon atoms colored violet). (B) Comparison of water accessibility of the spin label in the membrane-protein-water system and to a short peptide in aqueous solution. Radial distribution functions (RDFs) of water oxygen atoms around the nitroxyl oxygen were calculated from three independent 1 μs MD trajectories for both systems.

Note that in our case, the spin label was located in the polar region of the membrane and was accessible to both water and proton binding. If the pH-sensitive label is located deep within the protein molecule, proton diffusion through the protein's structural elements must be taken into account, similar to how this is done in proteins for electron transfer [48]

The data presented above were obtained using regular continuous wave (CW) EPR. We have also obtained data on the kinetics of PT in water using time-resolved EPR with fast CW detection [49], using a laser pulse for the excitation of the 2-NBA, and R4 as the proton acceptor (Figure 7). (The scheme for conducting this experiment is presented in Suppl. Mat.)

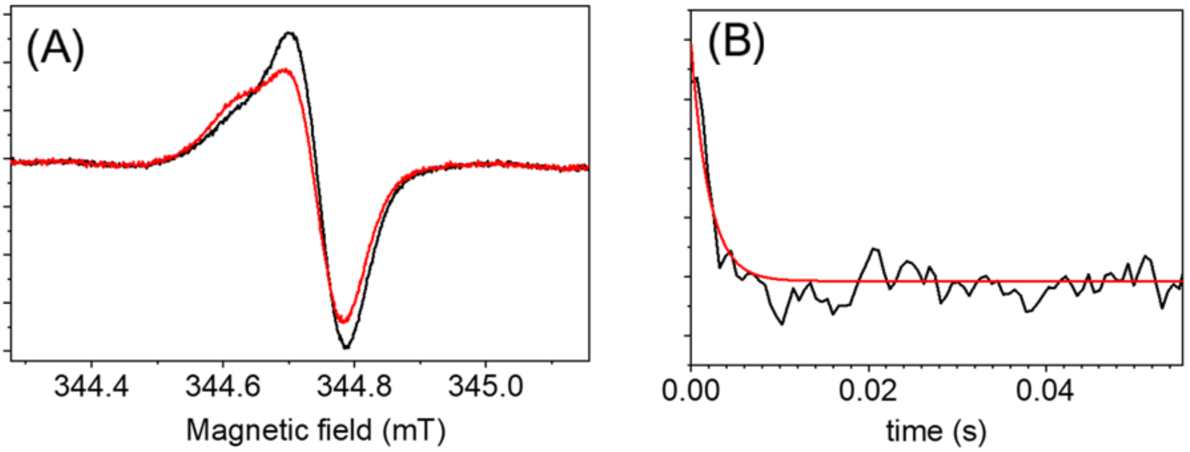

Figure 7. (A) X-band CW-EPR (high field hyperfine component) of the free base of radical R4 (c= 100 μM) in phosphate buffer 3 mM at pH 8.6 before irradiation (black line) and after irradiation

(red trace) of 2-NBA (c=1 mM). (B) Time dependence of the EPR intensity at 344.7 mT after a single laser flash (5 mJ/sh) at 355 nm. Microwave power was 6 mW , modulation amplitude of 0.07 mT (100 kHz), time constant and conversion time were 0.6 ms.

Previously we showed that nitroxyl radicals can be utilized to study electron transfer (ET) in chemical and biological systems [17,49,50]. pH-sensitive radicals can also be used for this purpose as well [49]. Thus, radicals of the type employed in the present study provide a unique approach to simultaneously studying ET and PT in proton-coupled electron transfer (PCET) systems [51-53].

Conclusion

In the present study pH-sensitive stable nitroxyl radicals, in conjunction with proton generation by photolysis of 2-NBA, were used, for the first time, to measure PT by EPR. It was found that water solutions with different chaotropic perturbation are characterized by different rate of PT, as expected. The approach was also used to show that in a water-glycerol mixture, which was a ‘glass’ at 160 K, PT most likely occurs by a tunneling mechanism. The methodology could also be applied for studying PT in a protein (as we demonstrated for bacteriorhodopsin) and membrane in which the pH-sensitive spin label was attached by site-directed spin-labelling.

Data Availability

All data used in this article is available in the following link:

https://weizmann.elsevierpure.com/en/datasets/9a1ffc70-729c-432a-9202-e7f051309c7b

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Conflicts of interest

There are no conflicts to declare.

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I.G., V.R., and I.K. did the synthesis, purification and characterization of used nitroxyl stable radicals. A.S. did the measurements and interpretation of PT at high field EPR spectrometer in ice. S.L. did molecular dynamic simulation. I.M. did determination of pK values of R2 in 6 M Gdn.HCl and 8 M urea.M.S. and T.E. did the spin labeling of mutants of bacteriorhodopsin and measurements PT in protein. N.F. did UV kinetics of photolysis of 2-NBA at different conditions. A.B. did time resolved experiments of R4 and R4 pK determination in Gdn.HCl solution. R.C. and L.W. did CW EPR investigation of kinetics of samples acidification at 2-NBA photolysis. A.B., M.S., R.C. and L.W. did the writing and editing of the manuscript. A.B. and L.W. conceptualization, supervision.

Author information

Authors and Affiliations

Department of Chemistry, University of Padova, Italy

Antonio Barbon

Max Planck Institute for Chemical Energy Conversion, Mülheim an der Ruhr, Germany

Anton Savitsky,

Novosibirsk Institute of Organic Chemistry, Novosibirsk, Russian Federation

Igor Grigoriev, Vladimir Reznikov, Igor Kirilyuk

Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel

Sofya Lushchekina

Department of Molecular Chemistry and Materials Science, Weizmann Institute of Science, Rehovot, Israel

Ilya Moroz, Tamar Eliash, Noga Friedman, Mordehai Sheves

Department of Chemical Research Support, Weizmann Institute of Science, ` ````Rehovot, Israel`

Raanan Carmieli

Department of Chemical Research Support and Department of Brain Research, Weizmann Institute of Science, Rehovot, Israel

Lev Weiner

Authors for corresponding:

Lev Weiner: leweiner1950@gmail.com

Antonio Barbon: antonio.barbon@unipd.it