pH-sensitive vibrational probe reveals a cytoplasmic protonated cluster in bacteriorhodopsin

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Infrared spectroscopy has been used in the past to probe the dynamics of internal proton transfer reactions taking place during the functional mechanism of proteins but has remained mostly silent to protonation changes in the aqueous medium. Here, by selectively monitoring vibrational changes of buffer molecules with a temporal resolution of 6 μs, we have traced proton release and uptake events in the light-driven proton-pump bacteriorhodopsin and correlate these to other molecular processes within the protein. We demonstrate that two distinct chemical entities contribute to the temporal evolution and spectral shape of the continuum band, an unusually broad band extending from 2,300 to well below 1,700 cm−1. The first contribution corresponds to deprotonation of the proton release complex (PRC), a complex in the extracellular domain of bacteriorhodopsin where an excess proton is shared by a cluster of internal water molecules and/or ionic E194/E204 carboxylic groups. We assign the second component of the continuum band to the proton uptake complex, a cluster with an excess proton reminiscent to the PRC but located in the cytoplasmic domain and possibly stabilized by D38. Our findings refine the current interpretation of the continuum band and call for a reevaluation of the last proton transfer steps in bacteriorhodopsin.

Proton transfers are one of the most ubiquitous chemical reactions in living organisms. Central to cellular bioenergetics are vectorial proton transfer reactions, conducted by light- and redox-driven proton-pumping membrane proteins (1, 2). Among the techniques sensitive to the protonation state of chemical groups in proteins, IR difference spectroscopy is well-suited for tracing intraprotein proton transfer reactions due to its intrinsic high temporal resolution, straightforward applicability to membrane proteins, and exquisite sensitivity (3–7). However, IR difference spectroscopy has not yet been able to monitor the dynamics of proton release from proteins into the surrounding aqueous phase, resulting in the need for complementary techniques to provide a comprehensive picture of vectorial proton transport. Protonation changes in the surrounding aqueous medium have been mostly probed in the visible range using pH-indicating dyes. These experiments require diluted and very weakly buffered protein solutions (8–11), in contrast to the well-buffered and highly concentrated samples used in IR spectroscopy (4, 12). It was soon realized that under low concentration of mobile buffers the released protons remain temporally trapped along the membrane surface, being detected by dye molecules in the bulk phase with a delay of ~0.5–1 ms (8, 9, 13, 14). Dyes covalently attached to the protein surface do show a fast response to proton release (10, 15–17), but complications arise because covalently attached dyes respond also to polarity changes at the protein surface (10, 15). Furthermore, their response time may depend on the distance to the proton release site (16, 17). Kinetic differences for proton detection have been observed even for dyes covalently bound to neighboring sites (15). As a final drawback, proton release/uptake kinetics may be altered whenever genetic engineering is needed to introduce a site for selective labeling (15, 17).

It has been known for more than 30 years that mobile buffer molecules accelerate the migration of protons from the surface to the bulk phase (9, 14) by collisional proton transfer (18, 19). Their chemical functionality as “proton shuttles” renders buffer molecules ideal probes for detecting proton release/uptake events by vibrational spectroscopy. A further advantage of buffer molecules over pH-indicating dyes is their high solubility and compatibility with biological samples at even molar concentrations, especially for the so-called Good’s buffers (20). A high buffer concentration ensures a well-controlled pH value during the entire length of the experiment and, more importantly, a fast response of the buffer to pH changes (e.g., proton release/uptake from proteins). As a drawback, extinction coefficients are typically 100 times smaller in the IR than in the visible range, which makes the use of buffer molecules as pH-sensitive probes technically more challenging than of pH-sensitive dyes.

Berthomieu and Hienervadel (21) reported on the use of buffer molecules to detect light-induced release of protons from photosystem II under steady-state conditions by FTIR difference spectroscopy. Experiments at the same pH using two different buffer molecules (e.g., phosphate vs. Tris buffer) were used to cancel overlapping protein contributions and to resolve buffer-only spectral changes (21). This methodology was recently improved for tracing intraprotein proton transfers.

Significance

The vectorial transport of protons across membranes by pumps is central to cellular bioenergetics. A persistent problem in their study is the technical unfeasibility to simultaneously resolve the dynamics of all the relevant proton transfer steps by the same method, that is, those within the protein as well as those involving protonation changes of the aqueous medium, currently relying on complementary methods to map both. Here, we solved this limitation and monitored both internal and external protonation changes during the proton-pump mechanism of bacteriorhodopsin by time-resolved infrared spectroscopy. Our findings reveal inconsistencies with the proton uptake mechanism accepted for the last 25 years, highlighting the need for simultaneous and comprehensive monitoring of protonation changes to resolve the molecular mechanism of ion pumps.

Infrared spectroscopy was used to probe the dynamics of internal proton transfer reactions taking place during the functional mechanism of proteins but has remained mostly silent to protonation changes in the aqueous medium. Here, by selectively monitoring vibrational changes of buffer molecules with a temporal resolution of 6 μs, it was possible to trace proton release and uptake events in the light-driven proton-pump bacteriorhodopsin and correlate these to other molecular processes within the protein. The technique allowed to demonstrate that two distinct chemical entities contribute to the temporal evolution and spectral shape of the continuum band, an unusually broad band extending from 2,300 to well below 1,700 cm−1. The first contribution corresponds to deprotonation of the proton release complex (PRC), a complex in the extracellular domain of bacteriorhodopsin where an excess proton is shared by a cluster of internal water molecules and/or ionic E194/E204 carboxylic groups. The second component of the continuum band was assigned to the proton uptake complex, a cluster with an excess proton reminiscent to the PRC but located in the cytoplasmic domain and possibly stabilized by D38. These findings refine the current interpretation of the continuum band and call for a reevaluation of the last proton transfer steps in bacteriorhodopsin.

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by performing experiments with natural and perdeuterated buffer molecules (22, 23). Further examples in the literature on the use of buffers as vibrational pH probes are scarce (21–26) and in all cases limited to steady-state experiments unable to provide information on protonation dynamics. A relevant example to test the potential of buffer molecules to probe the dynamics of proton release and uptake events is the light-driven proton-pump bacteriorhodopsin (BR) (27). This well-known transmembrane protein powers halophilic archaea under low oxygen tension (28). Its photocyclic reaction, triggered by photoisomerization of the retinal from all-trans to 13-cis, comprises a series of quasi-stable states, denoted as K, L, M, N, and O intermediates (29, 30), whose interconversion and decay to the initial dark state is, under most experimental conditions (30, 31), adequately described by a sequential model including back-reactions (Fig. 1A). Compelling evidence has been presented for the existence of at least two M (32, 33) and two N intermediates (34, 35).

Specific molecular events occur during the transition between intermediates (7, 36), such as proton transfer reactions (Fig. 1A and B). The widely accepted proton pumping mechanism of BR involves a minimum of five proton transfer reactions (Fig. 1B), leading to the net transport of one proton from the cytoplasmic (CP) to the extracellular (EC) side per photocycle (7, 37).

Gerwert and coworkers (38, 39) showed that the proton release complex (PRC), the elusive group releasing a proton to the EC medium in BR (Fig. 1A), was characterized by an unusually broad band extending from ~2,300 cm⁻¹ to well below 1,700 cm⁻¹ (Fig. 1C), known as the continuum band. This broadband feature was assigned to a PRC consisting of a protonated water cluster (39) or, more generally speaking, to a local area network (LAN) of H-bonded internal water molecules sharing an excess proton (40, 41) and stabilized by E194 and E204 (39). Similarly broad continua are predicted and observed for excess protons in liquid water (42–44), and reasonable spectral agreement with experiments has been reached in simulations placing an excess proton in the water molecules of the PRC (41). However, a PRC consisting of a proton shared by the ionic side chains of E194 and E204 can also reproduce the experimental continuum band (45), leading to a still open dispute about the precise chemical nature of the PRC (46). As a recent twist, Gerwert and coworkers (47) proposed that E194 is the actual terminal proton release group. If this is correct, the rise of the continuum band should precede the release of protons to the EC surface, a prediction that remains untested. Late intermediates in the photocycle of BR (from M to O; Fig. 1A) decay in an equilibrated mixture to the initial dark state (31, 48), a situation that considerably complicates studying late proton transfer events and, consequently, the proton uptake process. Established already in 1975 (49, 50), proton uptake was initially assigned either to the decay of the M or to the decay of the O intermediate (9, 51). Fifteen years later it was reassigned to the N-to-O transition when it was shown that D96 reprotonates in the N-to-O transition in a pH-dependent manner (35, 52, 53). Later results indicated that the reprotonation of D96 from the external medium might be assisted by several charged residues at the CP surface acting as a proton antenna (54, 55) with a prominent role for D38 (55).

In the present work we have traced the dynamics of proton release and uptake during the photocycle of BR by time-resolved step-scan FTIR spectroscopy. As a pH-sensitive vibrational probe we used MES and its perdeuterated form, MESd₁₂ (23). We exploited this technical achievement to scrutinize the current models of proton pumping by BR, in particular the proton release and uptake steps.

**Results and Discussion**

We prepared films of purple membranes (PMs), hydrated at 99% relative humidity. A representative absorption spectrum is shown in Fig. 2, from where we quantified the molar ratio of water/MES/BR molecules in the hydrated film to be 1,570/18.1/1, using the experimentally obtained molar IR absorption spectra of BR in PMs, MES, and liquid water (Experimental and Fig. 2). The above spectral decomposition also provides the molar ratio of the basic and acidic forms of MES, from where the pH of the hydrated film was derived to be 6.25 (close to the pH of 6.30 of the mother solution). **SI Appendix, Fig. S1** provides a representative absorption spectrum of a film containing MESd₁₂.

The hydration level attained in our experiments, ~1,600 water molecules per BR or ~0.8 g water/g (protein + lipid) when considering the composition of the purple membrane (56), is comparable to the average composition found in cells, ~2.3 g water/g biomolecules (57). These conditions are also close to those used in molecular dynamics simulations: 12,000–6,000 water molecules per BR molecule and ~1.5–4.9 g water/g (protein + lipids) (58, 59). In contrast, experiments carried out in solution using pH-sensitive dyes commonly contained ~5 × 10⁻⁸ water molecules per BR, or ~3,000 g water/g (protein + lipids) (9, 10, 17, 60).

The formal concentration of MES in the hydrated films, ~600 mM (~90 water molecules per buffer molecule), is ~10⁻⁴-fold higher than commonly used for pH-indicator dyes (9, 10, 17, 60). At this buffer concentration and pH any excess proton at the surface of BR is expected to protonate the basic form of MES with a pseudo first-order time constant of ~100 ns, which is calculated on the basis of the second-order rate constant of 1.8 × 10⁻⁴ M⁻¹s⁻¹ for MES protonation (14). This response time, even

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**Fig. 1.** Proton transfer reactions during the photoreaction of BR. (A) Basic photocycle scheme and (B) proton transfer steps overlaid with the dark-state structure (Protein Data Bank ID code 1c3w, ref. 97): (1) from the protonated Schiff base (SB) of the retinal chromophore to ionic D85; (2) from the PRC to the EC medium; (3) from D96 to the retinal SB; (4) from the CP medium to ionic D96, and finally (5) from D85 to the PRC via D212 (66, 84). (C) Time-resolved step-scan FTIR difference spectra after 10-ns laser pulse excitation. (D) Expanded absorption changes in the 2,000- to 1,700-cm⁻¹ region, with contributions from the C=O stretch of D85, D96, and D115, as well as from the continuum band, assigned to the PRC.
deprotonation and reprotonation of the Schiff base in the 13-cis conformation of the retinal and, among other bands, can be used as a reporter for the formation and decay of the K, L, and N intermediates (67).

Transient Protonation Changes of MES Buffer. Among other IR features, protonation of MES buffer is characterized by a negative narrow band at 1,112 cm\(^{-1}\) (23), with a moderate change in extinction coefficient of \(\Delta\varepsilon = 210 \text{ M}^{-1}\text{cm}^{-1}\) (SI Appendix, Fig. S2). A small negative band is indeed observed at 1,112 cm\(^{-1}\) in the light-induced IR difference spectrum of BR 370 \(\mu\)s after photoexcitation (Fig. 3A, blue), a time when a proton is released from the protein to the medium (7). In experiments where

if approximate, is sufficiently fast to ensure that the kinetics for proton release in BR can be traced without any significant delay.

Internal Protonation Changes. The photoreaction of BR was triggered by a 10-ns laser pulse of 532 nm, and time-resolved step-scan FTIR spectroscopy was used to record transient absorption changes in the range of 2,200–850 cm\(^{-1}\) (Fig. 1C). Kinetics for the protonation changes of internal groups, involved in proton transfer reactions in the photocycle of BR, can be retrieved from such data as shown before (48, 61) and briefly described below.

The rise of the positive band at \(\sim 1,762 \text{ cm}^{-1}\) reports on protonation of D85 (Fig. 1D). A shift of the carboxylic C=O stretching frequency from 1,762 to 1,755 cm\(^{-1}\) occurs in the M-to-N transition (62). Deprotonation of D85 is indicated by the decay of the positive band at \(\sim 1,755 \text{ cm}^{-1}\) (29, 61). D96 is protonated in the dark state of BR, with its carboxylic C=O vibration absorbing at \(\sim 1,742 \text{ cm}^{-1}\) (63). The negative band at 1,739–1,745 cm\(^{-1}\) in Fig. 1D is mainly caused by transient H-bonding changes of D96, which shifts the C=O vibration to 1,748 cm\(^{-1}\) in the L intermediate (64) and to 1,736 cm\(^{-1}\) in the M intermediate (63). Deprotonation and reprotonation of D96 contributes to the kinetics of the negative band at \(\sim 1,742 \text{ cm}^{-1}\) in the milliseconds (Fig. 1D) although its contribution is low at pH 6.25 because the apparent pK\(_a\) of D96 in the N intermediate is \(\sim 7.1\) (34, 65). Consequently, at pH 6.25 the fraction of the N intermediate with deprotonated D96 (N\(_1\) substate) is expected to be only around 12%. Deprotonation of D212 from D85 gives a positive band at \(\sim 1,713 \text{ cm}^{-1}\), although it is observable only at pH 4 (66). Remaining spectral changes in the carboxylic region are mostly attributable to H-bonding changes of D115 (Fig. 1D), with its carboxylic C=O vibration absorbing at \(\sim 1,733 \text{ cm}^{-1}\) in the dark state (63). The occurrence of the very broad negative continuum band at frequencies above 1,770 cm\(^{-1}\) in Fig. 1D is assigned to the deprotonation of the PRC (39) and decays upon dark-state recovery (38). The positive band at \(\sim 1,187 \text{ cm}^{-1}\) (Fig. 1C, arrow) can be used to monitor the
deuterated MES$_{d12}$ was used (Fig. 3A, red) the negative band at 1,112 cm$^{-1}$ disappears and a positive band at 1,115 cm$^{-1}$ appears (Fig. 3A), the latter characteristic for protonation of MES$_{d12}$ (23) (SI Appendix, Fig. S2). Spectral changes associated with protonation of MES during the photocycle of BR become fully accessible by calculating the double difference spectrum between experiments done in MES and in MES$_{d12}$, after appropriate scaling to cancel the spectral response of the protein (Fig. 3B, red spectrum).

The resulting absorption changes in the double difference spectra, 2.5 $\times$ 10$^{-4}$ for the most intense band at 1,113 cm$^{-1}$ (Fig. 3B, red spectrum), are 20–100 times smaller than typically measured with pH-indicating dyes in the visible (9, 10, 15, 60). Nevertheless, most of the bands in the double difference spectrum are clearly resolved despite their low intensity. Most importantly, the resulting spectral changes are identical to the double difference spectrum between protonation of MES and MES$_{d12}$ induced by pH changes in the solution (Fig. 3B, green spectrum), with band positions within 1-cm$^{-1}$ agreement for nearly all bands. Given the sensitivity of molecular vibrations to their environment, this observation supports the expectation that MES molecules protonate and deprotonate in the aqueous phase, and not at the protein surface or its interior.

We monitored the protonation changes of MES, reporting on proton release and uptake from BR, from the area of the most intense negative bands at 1,113 cm$^{-1}$ in the double difference spectra, as well as from the joint area of the most intense positive bands at 1,093 and 1,082 cm$^{-1}$ (Fig. 3C, light-blue and orange traces, respectively). Both kinetic traces are very similar, as expected, although monitoring the area of the 1,113-cm$^{-1}$ band clearly gives superior signal-to-noise ratio (Fig. 3C, compare light-blue and orange traces) due to its higher intensity (SI Appendix, Fig. S2). Monitoring the area of the 1,113-cm$^{-1}$ band is also more robust to scaling errors in the subtraction of protein bands, as these contribute less than 15% to the total area (SI Appendix, Fig. S3B).

**Kinetics of Proton Release and Uptake.** Fig. 4A reproduces the temporal evolution of the MES-minus-MES$_{d12}$ band at 1,133 cm$^{-1}$ during the photocycle of BR. Our kinetic analysis was based on reconstructing the lifetime distributions using the maximum entropy method (68), with the benefit that the number of exponentials is not presumed a priori, as in traditional fitting approaches. Note that positive bands report on MES protonation (proton release by the protein) and negative bands on MES deprotonation (proton uptake by the protein). This convention is kept throughout the entire paper.

**Proton release.** The release of the protons to the medium detected by covalently attached dyes has been previously reported to be monoeponential, with $\tau \approx 75$ ms (16, 17, 38). In good agreement, the analysis of the kinetics for MES protonation revealed one component for proton release with a mean time constant of 80 ± 4 ms (Fig. 4B, dashed red trace). Confidence intervals are given here and elsewhere as ± two SDs from Monte Carlo simulations (Experimental). However, this component was broad and slightly asymmetric, suggesting the presence of unresolved subcomponents. Indeed, increasing the resolution of the reconstructed lifetime distribution leads to the resolution of two statistically significant components for proton release with $\tau = 52 ± 10$ ms and 150 ± 40 ms (Fig. 4B, red trace and SI Appendix, Fig. S4) and relative amplitudes of 67 ± 12% and 33 ± 12%, respectively.

We further confirmed that proton release is biexponential by reanalyzing transient absorption changes in solution from fluorescein covalently attached to K129 (10) (SI Appendix, Fig. S5), an amino acid located in the EC surface (Fig. 1B). Two components for proton release are clearly resolved in the corresponding lifetime distribution (Fig. 4B, Inset, blue trace), with $\tau = 59 ± 3$ ms (75 ± 4%) and 200 ± 20 ms (25 ± 4%). These results are in good agreement with those determined by MES (discussed above), in particular when taking into account the slight differences in temperature and pH between both experiments (see Fig. 4 legend).

**Proton uptake.** The analysis of the protonation kinetics of MES shows that proton uptake kinetics in BR proceeds mostly in a monoeponential fashion. The main decay component is resolved at $\tau = 4.3 ± 0.4$ ms (Fig. 4B, red trace). This time constant agrees with the last time constant of the photocycle obtained by global exponential fitting ($\tau = 4.4 ± 0.1$ ms, discussed above), when an equilibrated mixture of the M, N, and O intermediates decays to the dark state.

The lifetime distribution corresponding to protonation changes of MES also resolved a negative band at $\tau = 8 ± 4$ ms (Fig. 4B) (i.e., a very early uptake of protons by BR). We can reasonably discard that such a component is an artifact caused by the limited time resolution of our FTIR experiments because a similar negative component at $\tau = 10 ± 2$ ms is also resolved when analyzing nanosecond-resolution fluorescein experiments (Fig. 4B, Inset). Indeed, a fast response of covalently attached fluorescein has been observed before (38, 69) but tentatively assigned to polarity changes, an unlikely interpretation in view of its detection by MES. Thus, we conclude that some
uptake of protons occurs in the photocycle of BR as early as in few microseconds.

**Protonation of D85 vs. Proton Release.** Although protonation of D85 and proton release take place at spatially distinct sites, the two reactions appear as simultaneous events under most conditions (36), the first triggering the latter by a domino effect involving fast H-bonding rearrangements and reorientation of R82 (70). Now that it is possible to monitor both events in a single experiment, we resolved a small but clear temporal delay between protonation of D85 and proton release (Fig. 5A). For further insights we compared their lifetime distributions (Fig. 5B), displayed such that positive bands correspond to D85 protonation (blue) and to proton release (red). Protonation of D85 occurs in three phases (17, 60) with \( \tau = 4 \pm 1 \mu s \) (10 \( \pm 6\% \)), 38 \( \pm 3 \mu s \) (55 \( \pm 6\% \)), and 115 \( \pm 10 \mu s \) (35 \( \pm 6\% \)). The time constants of the second and third components are slightly faster than for proton release: 38 \( \pm 3 \mu s \) vs. 52 \( \pm 10 \mu s \) and 115 \( \pm 10 \mu s \) vs. 150 \( \pm 40 \mu s \), but the deviations are modest when considering the statistical uncertainty (Fig. 5B). The most notable difference is that the first step for protonation of D85 (4 \( \pm 1 \mu s \)) is not associated with a proton release event, but rather with a proton uptake event (8 \( \pm 4 \mu s \)).

**Kinetics of the Continuum Band vs. Proton Release.** If the continuum band is a spectral signature of the group releasing a proton to the EC medium, then its rise should display kinetics identical to those of proton release. However, a meaningful kinetic comparison has not been possible yet (38).

We analyzed the temporal evolution of the continuum band by integrating the absorption changes from 1,950 to 1,800 cm\(^{-1}\) (Fig. 6A, black crossed dots). A significant net contribution to this area from temperature changes of water molecules during the photocycle was discarded by control experiments using the E204Q mutant (SI Appendix, Fig. S6B), a variant that lacks absorption changes in this frequency range (38). The corresponding lifetime distribution of the continuum band shows two rising components with \( \tau = 42 \pm 3 \mu s \) and 150 \( \pm 6 \mu s \) and relative amplitudes of 63 \( \pm 4\% \) and 37 \( \pm 4\% \) (Fig. 6B, red trace). These two components are basically indistinguishable at our current statistical uncertainty from those of proton release detected by MES, with \( \tau = 52 \pm 10 \mu s \) (67 \( \pm 12\% \)) and 150 \( \pm 40 \mu s \) (33 \( \pm 12\% \)). These results are fully consistent with the PRC being the terminal group releasing a proton to the EC medium (39).

The lifetime distribution of the continuum band shows an additional rise with \( \tau = 1.5 \pm 0.1 \) ms, not associated with any proton release/uptake event (Fig. 6B, compare red and blue traces). The presence of this kinetic component has been noticed before (38). Initially, it was assigned to a LAN of water molecules assisting the proton transfer from D96 to the SB (38), but such a neutral LAN was later shown to generate a very broad absorption band above 2,550 cm\(^{-1}\) instead (71, 72). Its time evolution clearly differs from the M intermediate (compare Figs. 5A and 6A), and to a lesser extent from the O intermediate (Fig. 6D), being most similar to that of the N intermediate (Fig. 6C). Because of the minor accumulation of the N\(_1\) intermediate at pH 6.25, it is reasonable to associate the rise of the second component of the continuum band with the formation of the N\(_2\) intermediate (65). The accumulation of the N\(_2\) intermediate decreases with increasing pH (34, 65). Likewise, the amplitude of the continuum band rising with \( \tau = 1.5 \) ms has been shown to decrease as the pH increases (73).

It is finally noted that both phases of the continuum band recover with \( \tau = 4.5 \pm 0.1 \) ms (Fig. 6B). This time constant is in close agreement with the time constant for proton uptake, 4.3 \( \pm 0.4 \) ms (Fig. 4B), as well as with the recovery of the dark state: \( \tau = 4.4 \pm 0.1 \) ms (Fig. 7A).

**Two Spectrally Distinct Chemical Groups Contribute to the Continuum Band.** To obtain the spectral features associated to the two kinetic components of the continuum band we performed global exponential fitting of the time-resolved FTIR spectra. The derived amplitude spectra (Fig. 7A), or decay-associated spectra (DAS),
provide the spectral changes taking place with a specific time constant (74). The components DAS-2 and DAS-3, with $\tau = 44$ and 127 $\mu$s, respectively, correlate with the release of a proton from the protein and its acceptance by the MES buffer in the medium. Both spectra display a continuum band with a similar shape (Fig. 7A): The absorption changes rise in intensity from 2,200 cm$^{-1}$ to 2,000 cm$^{-1}$ and remain constant from 2,000 cm$^{-1}$ to 1,800 cm$^{-1}$. DAS-5, with $\tau = 1.6$ ms, shows a clear continuum band (Fig. 7A), even though no proton is released or taken up with this time constant (Fig. 6A and B). The continuum band from DAS-5 continuously rises in intensity from 2,200 to 1,800 cm$^{-1}$ (Fig. 7A), spectrally differing from the continuum band characteristic for DAS-2 and DAS-3 (Fig. 7A). The above-mentioned spectral differences between the continuum bands of DAS-2 and DAS-5 are highly reproducible (Fig. 7B). We conclude that the continuum band observed in the DAS-5 has a molecular origin other than the PRC. Thus, the negative rise of the continuum band with $\tau = 1.5$ ms presumably represents the deprotonation of a newly described protonated LAN, to which we will refer as the proton uptake complex (PUC) for reasons which will be justified below.

The PUC and Reprotonation of D96. To figure out the functional role of the PUC in the proton-pumping mechanism of BR we studied in detail the spectrum of DAS-5 (Fig. 7A). The negative band at 1,187 cm$^{-1}$, characteristic for the C–C stretch of protonated 13-cis retinal, is a hallmark of the reprotonation of the SB of 13-cis retinal (i.e., for the formation of the N intermediate from the M intermediate) (29, 67). The pH of the sample is 0.85 units below the apparent pK of D96 in the N intermediate, favoring accumulation of the N$_2$ substate, with reprotonated D96 (34, 65). Consistently, a positive band at 1,742 cm$^{-1}$ from the deprotonation of D96 is hardly observable in DAS-5 (Fig. 7A) even when performing step-scan experiments at an increased resolution of 4 cm$^{-1}$ (SI Appendix, Fig. S7). Incidentally, DAS-5 also shows retinal bands at 1,506 and 1,170 cm$^{-1}$ characteristic for the formation of the O intermediate (53, 75), consistent with the known equilibration of the N and O intermediates (76). In summary, DAS-5 represents the spectral differences associated with the net formation of the N$_2$ and O intermediates from the M intermediate.

How to conceive the formation of the N$_2$ intermediate with $\tau \approx 1.5$ ms with a reprotonated D96, when proton uptake from the medium occurs with $\tau \approx 4.5$ ms? Also, if the continuum band with $\tau \approx 1.5$ ms reports on the deprotonation of a protonated LAN (the PUC), where is the corresponding proton transferred to? To answer these two questions we propose that ionic D96 gets a proton in the N$_2$-to-N$_2$ transition from the PUC, not from the CP medium as currently accepted. Finally, the PUC is reprotonated in the N$_2$-to-O transition, taking a proton from the CP medium (Fig. 7C).

General Discussion and Conclusions

The use of buffer molecules (weak acids) to trace protonation changes in the aqueous medium during the functional mechanism of proteins solves some of the drawbacks present in the use of pH-indicating dyes (see the Introduction). In particular, buffer molecules can theoretically respond to protonation changes in the medium in submicroseconds (14), thanks to their high concentration under experimental conditions typical for IR spectroscopy.

Fig. 7. Spectral signatures for the PRC and the PUC and revised model for proton transfer reactions in BR. (A) DAS obtained from global exponential fitting analysis of time-resolved FTIR experiments at 8 cm$^{-1}$ resolution using MES and MES$_{d_2}$ as buffer (blue and red continuous lines, respectively). The region between 2,150 and 1,800 cm$^{-1}$ is 20-fold magnified (dashed lines). The DAS with $\tau = 4.4$ ms is scaled by 0.5 for display purposes. (B) DAS-2 and DAS-5 for five independent experiments, including their average (back line). The asterisk marks a band originated from MES$_{d_2}$. (C) Extended photocycle scheme of BR, integrated into the dark-state structure (B7). The precise chemical nature and location of the PUC remains elusive, but its deprotonation is characteristic for DAS-5 (8).
Although we have not been able to test this last prediction experimentally, we can conclude that buffer molecules can respond to protonation changes in at least 8 μs (Fig. 4B). Because pH-indicating dyes are weak acids they are able to shuttle protons from the surface to the bulk (8). However, their typical high extinction coefficient in the visible often restricts their concentration to <100 μM (8–11), while their low concentration makes them inefficient accelerators for proton migration in practice.

The dynamics of proton release and uptake in BR are apparently more complex than previously described: Proton release cannot be described by a single exponential time course as reported before (16, 17, 38) but proceeds in at least two phases with time constants of τ = 50 μs and 150 μs (Fig. 4B). Proton release lags protonation of D85 (Fig. 5), supporting photocycle models that place proton release during the transition between two M intermediates (37, 77).

Although proton uptake is predominantly monoexponential, τ ≈ 4.5 ms, we resolved a very early proton uptake event taking place before 10 μs (Fig. 4B). It is conceived that this early proton uptake arises from Bohr protons. Nanosecond IR experiments (78, 79) at different pH values will be required in the future to characterize the exact timing of this early proton uptake process, as well as to understand its potential role in the photocycle and to assign the residue(s) responsible for it.

Despite the current consensus that the continuum band in BR arises from the deprotonation of the PRC, it was yet to be experimentally confirmed whether proton release to the external medium tallies the rise of the continuum band. Due to the strict comparison of the kinetics of proton release (probed by MES buffer molecules) with the kinetics of the continuum band we have shown here that both processes indeed proceed simultaneously (Fig. 6). It has been recently proposed that the PRC protonates E194, with the latter residue being the actual terminal proton release group (47). From the discrepancy between the faster component for proton release (52 ± 10 μs) and the faster rise of the continuum band (42 ± 3 μs) we can state that any intermediary proton acceptor/donor group in between the PRC and the external medium can hold the proton for less than 10 ± 10 μs. Given their kinetic similarity we can reasonably exclude that a metastable proton-accepting group exists in between the PRC and the external medium.

Comparison of the dynamics of the continuum band with proton release/uptake detected by buffer molecules indicates that the continuum band consists of two independent kinetic (Fig. 6) and spectral (Fig. 7 A and B) contributions. As elaborated above, the first contribution to the continuum band corresponds to deprotonation of the PRC, as shown by site-directed mutagenesis studies (39) and further confirmed herein by its temporal coincidence with proton release (Fig. 6 A and B). Spectral calculations reproduce the continuum absorption between 2,300 and 1,800 cm⁻¹ when, independently of molecular details, the PRC consists of a protonated LAN (41, 45, 46). Thus, the assignment of the continuum band to a protonated LAN at the PRC is well supported by experiments and simulations.

We have resolved a second continuum band contributing to the 2,300- to 1,800-cm⁻¹ range whose kinetics does not correlate with proton release (Fig. 6C). We have tentatively assigned the second component of the continuum band to a protonated LAN, given its spectral similarity to the continuum band from the PRC (Fig. 7). Further support for this assignment comes from the results that indicate the existence of an intermediary group/complex that can act as a proton donor for D96 and as a proton acceptor from the CP medium (Figs. 6 and 7); the PUC. The kinetics of the second contribution of the continuum band fits the expected kinetics for the PUC, consistent with its assignment to a protonated LAN. In analogy to the PRC, the protonated LAN of the PUC may involve water molecules and/or several amino acid side chains sharing a proton.

Neutral LANs also exist in BR, and their spectral contributions during the photocycle should be discussed. The pentagonal cluster (protonated SB, the charged groups D85, D212, and R82, and three water molecules) has been studied in detail in IR difference spectroscopy (80). It shows notably broad negative bands from strongly H-bonded O–H and N–H stretches, but these are centered at ≥ 2,800 cm⁻¹ and display a negligible contribution at frequencies below 2,600 cm⁻¹ (81, 82). A chain of neutral water molecules formed between the SB and D96 in the M intermediate has been associated with a broad positive band in the 2,750- to 2,550-cm⁻¹ region (71, 72). However, neither of these two neutral LANs contributes significantly to the 2,300- to 1,800-cm⁻¹ region, which seems restricted to protonated LANs.

We anticipate that the identification of the groups comprising the PUC will require further work. Reasonable candidate residues are those that may alter the N₅/N₂ equilibrium when mutated (34), or the proton uptake kinetics (55). Among the latter, the most evident candidate is D38. Consequently, we performed preliminary time-resolved IR experiments on D38R using a tunable quantum cascade laser as an IR monochromatic source (79, 83), covering the spectral range from 1,800 to 1,700 cm⁻¹. Remarkably, the kinetics of the continuum band for the D38R variant lacks the millisecond negative rise present in WT (SI Appendix, Fig. S8), indicating that the PUC might, indeed, be formed upon mutation of D38. More detailed studies will be needed, though, as D38R also shows altered kinetics for the late intermediates of the photocycle (M, N, and O) (55), which by itself could explain the apparent absence of the continuum band from the PUC.

In the current view of the proton transfer reactions in BR, D96 is reprotoanted from the CP medium (Fig. 1C), either in the N-to-O transition (77) or in the N₁₉-N₂ transition (37). Instead, we suggest that D96 accepts a proton from the PUC in the N₁₉-to-N₂ transition, and the PUC is reprotoanted from the CP medium in the N₂-to-O transition (Fig. 7D). A piece of evidence is provided by the observation that proton uptake from the CP medium (τ ≈ 4.5 ms) considerably lags the formation of the N₂ intermediate, which takes place with τ ≈ 1.5 ms (Figs. 4B, 6C, and 7A). However, the PUC deprotoanted with τ ≈ 1.5 ms and reprotoanted with τ ≈ 4.5 ms (Figs. 6B and 7A). Thus, our results refine the last steps of the proton-pumping mechanism of BR. While the presence of the second continuum band is observed under conditions that prevent deprotoation of the PUC (low pH or exchange of critical residues), though with reduced intensity and delayed kinetics (39, 73). The continuum band disappears completely in the E194Q and E204Q variants (38, 39), implying that these mutations impair not only the PUC but also the PUC. E194Q and E204Q variants not only lack normal proton release but also show notable alterations in the late steps of the photocycle as well (66, 84), possibly explaining why these two mutations disturb the distant PUC.

We note that our findings might also contribute, revealing the chemical nature of the PRC, an issue that is still not settled (46). To scrutinize different potential arrangements of the PRC, simulated vibrational spectra of the PRC have been compared with the experimental difference spectrum of the continuum band (41, 45, 46), extracted at 300–400 μs after photoexcitation (see figure 2 in ref. 39 for an example). However, at 300–400 μs not only the PRC but also the PUC contributes to the measured continuum band (Fig. 6A). We have obtained the spectral signature of the continuum band associated with proton release by global exponential analysis (DAS-2, Fig. 7B). This is the experimental spectrum that should be ideally used in future comparisons with spectral calculations of the PRC.

In closing, we have demonstrated here that the time course of proton release and uptake accompanying the photocycle of BR can be monitored by recording time-resolved vibrational changes.
of natural and perdeuterated buffer molecules, which work as pH-sensitive vibrational probes. As proton transfer reactions often play a critical role in protein function, similar experiments could be applicable to other proteins.

Experimental
Preparation of Hydrated Films of BR. PMs containing WT BR, E204Q, or D38R were obtained from *Halobacterium salinarum* (85–87). The PMs were washed by centrifugation and resuspended in 3 mM MES and 2 mM NaCl at pH 6.3. Around 10–20 μl of this solution was placed on top of a BaF$_2$ window and dried under ambient humidity, followed by rehybridization in an atmosphere with 99% relative humidity, as previously described (12, 88). Equivalent films were prepared using MESS$_{12}$ (Cambridge Isotope Laboratories, Inc.) as a buffer, a molecule where all of the hydrogens of MES unchargeable in water are substituted by deuterium atoms. The final molar ratio of protein, buffer, and water was determined by fitting molar absorption spectra to the experimental absorption spectrum of the hydrated film (Fig. 2 and SI Appendix, Fig. S1). We also determined spectroscopically the pH in the hydrated film by applying the Henderson–Haselbalch equation, using the pk$_{a}$ for MES (6.06 at an ionic strength of 0.5 M and 25 °C (www.realdevices.com/Protein/BiologicalBuffers.html)) and the ratio between acidic and basic forms determined by IR absorption spectroscopy. The ionic strength of the film was calculated to be close to 1 M, minimizing complications from pH differences between the BR surface and the medium pH, which can be as high as 1.7 units at 10 mM and still near 1 unit at 100 mM (60), but only 0.2 units at 1 M ionic strength (89).

Step-Scan FTIR Spectroscopy. We performed time-resolved step-scan FTIR spectroscopy essentially as described before (12). Light-adapted BR films containing either the buffer MES or MESS$_{12}$, were excited by a 10-ns laser pulse (532 nm, 2 mJ/cm$^2$, 10 Hz) and time-resolved spectra were obtained at 6.25-μs temporal and 8-cm$^{-1}$ spectral resolution. Some additional experiments were performed at 4-μs spectral resolution as indicated. In each experiment, about 300–500 photo-reactions were averaged at each optical retardation of the interferogram.

Maximum Entropy Lifetime Distributions. Maximum entropy lifetime distributions were obtained from experimental time traces as described before (90). Briefly, a maximum entropy solution vector, $h$, was obtained minimizing the function $Q(h) = x^T(h) - s(h)$, where $s$ is the generalized Shannon-related entropy for solutions without sign restrictions (quantifying the simplicity of a solution), $x^T$ measures the agreement between the experimental and the predicted data, and $n$ is a scalar which balances both terms, known as the regularization parameter. Lifetime distributions of integrated detail are obtained as the value of $n$ is reduced, giving more weight to the description of the data over the simplicity of the solution. The optimum value for log$_{10}(n)$ was determined automatically using the L-curve method (91, 92). The maximum entropy lifetime distribution obtained in this way lacked enough detail for proton release/uptake measured with MES, and in this case, a modified maximum entropy algorithm was used. The maximum entropy lifetime distribution obtained in his way lacked enough detail for proton release/uptake measured with MES, and in this case, a modified maximum entropy algorithm was used. The optimum value of $n$ was estimated from the known isotropic extinction coefficient of light-adapted BR in the membrane plane from the extinction coefficient of the retinal chromophore: $e_{1}(570 \text{ nm}) = 82,000 \text{ M}^{-1} \text{cm}^{-1}$. This last value was estimated from the known isotropic extinction coefficient of BR determined in solution ($e_{1}(570 \text{ nm}) = 62,700 \text{ M}^{-1} \text{cm}^{-1}$ (93)), the degree of the electronic transition moment of the retinal to the membrane normal, $\theta$ = 69° (96), and the relations for axially oriented samples: $e_{1} = (e_{1} + 2e_{2})/3$ and $(e_{1} - e_{2})/(e_{1} + 2e_{2}) = 0.5(3\cos^{2}\theta - 1)$ (96).

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Kinetics of the Continuum Band. The kinetics of the continuum band was obtained by integrating the absorption changes from 1,950 to 1,800 cm$^{-1}$. The baseline of the time-resolved data showed oscillations of period ~1 ms (SI Appendix, Fig. S5), whose origin has been described before (93), hampering the analysis of the kinetics of the continuum band. We removed these oscillations by processing the spectra by singular value decomposition (SVD), discarding only 2 of the 147 SVD components (those concentrating the oscillation). Further details of this processing approach will be presented elsewhere.

Absorption Coefficients of MES Determined by Attenuated Total Reflection FTIR Spectroscopy. We prepared duplicate aqueous (H$_2$O) solutions of MES and perdeuterated MESS$_{12}$ at 50 mM and at 90 mM, with the pH value adjusted to 3.6 (adding HCl) and 8.5 (adding NaOH). The infrared absorption spectrum was recorded at 25 °C and 4-cm$^{-1}$ spectral resolution in a diamond attenuated total reflection (ATR) accessory with nine reflections (five of them facing the sample). We subtracted the absorption of water at the corresponding pH value to obtain the absorption spectrum of fully ionic and fully zwitterionic MES and MESS$_{12}$ (SI Appendix, Fig. S2A). After accounting for the concentration of MES and the calculated effective penetration depth in the ATR experiment (discussed below), the difference molar absorption coefficient spectra were determined for protonation of MES and for protonation of MESS$_{12}$, and the double difference molar absorption coefficient for protonation of MES-minus-protonation of MESS$_{12}$ (SI Appendix, Fig. S2B). The latter was mathematically converted to 8-cm$^{-1}$ resolution in the Fourier domain before its comparison with the time-resolved double difference IR spectra between BR/MES and BR/MESS$_{12}$ (Fig. 3B). We determined the effective penetration depth as a function of the wavenumber, $d_{p}(\tilde{v})$, experimentally (SI Appendix, Fig. S9). Briefly, the absorbance of liquid water (MilliQ quality, pH 7, 25 °C) was recorded with the ATR setup (four replicates on different days), and the $d_{p}(\tilde{v})$ was calculated from the concentration of water (55.34 M at 25 °C) and its published molar absorption coefficient (94).

Molar Absorption Coefficient of BR Determined by Transmission FTIR Spectroscopy. We measured the UV-visible and IR absorption of a hydrated film of BR in PMs in the absence of buffer, previously adjusting the solution to pH 7 (SI Appendix, Fig. S9). An iris with a diameter less than 4 mm (smaller than the probing light) was placed in front of the BaF$_2$ window to ensure that both the UV-visible and the IR radiation illuminated the same area. The sandwiched sample was placed normal to the light beam. Before the measurements the BR film was illuminated with an LED emitting maximally at 530 nm for 1 min for light adaptation. We estimated the IR molar absorption coefficient of light-adapted BR in the membrane plane from the extinction coefficient of the retinal chromophore: $e_{1}(570 \text{ nm}) = 82,000 \text{ M}^{-1} \text{cm}^{-1}$. This last value was estimated from the known isotropic extinction coefficient of BR determined in solution ($e_{1}(570 \text{ nm}) = 62,700 \text{ M}^{-1} \text{cm}^{-1}$ (93)), the degree of the electronic transition moment of the retinal to the membrane normal, $\theta$ = 69° (96), and the relations for axially oriented samples: $e_{1} = (e_{1} + 2e_{2})/3$ and $(e_{1} - e_{2})/(e_{1} + 2e_{2}) = 0.5(3\cos^{2}\theta - 1)$ (96).

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