Surface-bound optical probes monitor proton translocation and surface potential changes during the bacteriorhodopsin photocycle

(purple membrane/proton pump/pH indicator/proton transfer/flash spectroscopy)

JOACHIM HEBERLE AND NORBERT A. DENCHER

Hahn–Meitner–Institut, BENSC-N1, Glienicke Strasse 100, W-1000 Berlin 39, Federal Republic of Germany

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ABSTRACT Light-induced H^+ release and reuptake as well as surface potential changes inherent in the bacteriorhodopsin reaction cycle were measured between 10°C and 50°C. Signals of optical pH indicators covalently bound to Lys-129 at the extracellular surface of bacteriorhodopsin were compared with absorbance changes of probes residing in the aqueous bulk phase. Only surface-bound indicators monitor the kinetics of H^+ ejection from bacteriorhodopsin and allow the correlation of the photocycle with the pumping cycle. During the $L_{550} \rightarrow M_{412}$ transition the H^+ appears at the extracellular surface of bacteriorhodopsin. Surface potential changes detected by bound fluorescein or by the potentiometric probe 4-[[2-(di-*n*-butylamino)-6-naphthyl]vinyl]-1-(3-sulfo-propyl)pyridinium betaine (di-4-ANEPPS) occur in milliseconds concomitantly with the formation and decay of the N intermediate. pH indicators residing in the aqueous bulk phase reflect the transfer of H^+ from the membrane surface into the bulk but do not probe the early events of H^+ pumping. The observed retardation of H^+ at the membrane surface for several hundred microseconds is of relevance for energy conversion of biological membranes powered by electrochemical H^+ gradients.

A challenge in membrane research is the understanding of generation, maintenance, and utilization of electrochemical H^+ gradients. Especially the translocation of H^+ across integral membrane proteins and subsequent H^+ transfer into the aqueous bulk phase are less than well understood. Analysis of vectorial H^+ transfer reactions is hampered by strong interactions of H^+ with the membrane surface—i.e., with lipid head groups and exposed amino acids (1). These interactions will severely influence the rate of diffusion-controlled transfer of H^+ into the aqueous bulk phase (2). Therefore, it is necessary to monitor the kinetics of H^+ transfer directly at the membrane surface, e.g., close to the H^+ ejection site of an integral membrane protein (3).

Bacteriorhodopsin (BR) in the purple membrane (PM) of *Halobacterium halobium* is a very attractive system for the investigation of molecular steps in light-driven vectorial H^+ translocation. Although BR is an integral membrane protein (26,500 Da) with seven transmembrane α -helices, its structure is known at 3.5- to 7.8-Å resolution, including the approximate position of many of the 248 amino acids (4). From the position of the chromophore retinal (5, 6), bound to Lys-216, the location of the protonated nitrogen of the Schiff base that is part of the active center of this transport protein can be inferred. To correlate H^+ ejection kinetics with the corresponding spectroscopic intermediate(s) of the BR photocycle (7, 8), with protonation changes of amino acids (9–12), and with alterations of the tertiary structure of the protein in the vicinity of the chromophore (13, 14), H^+

transfer was monitored with the optical pH indicator fluorescein covalently linked to the extracellular surface of BR, at Lys-129. Concomitant surface potential changes were reflected by absorption changes of both fluorescein and the potentiometric dye 4-[[2-(di-*n*-butylamino)-6-naphthyl]vinyl]-1-(3-sulfo-propyl)pyridinium betaine (di-4-ANEPPS).

MATERIAL AND METHODS

The pH indicator fluorescein [5(6)-carboxyfluorescein succinimidyl ester (FSE) or fluorescein-5-isothiocyanate (FITC), Molecular Probes] was covalently conjugated (via a carboxamide or thiourea linkage, respectively) to the extracellular surface of BR (Fig. 1). To remove unbound fluorescein, the labeled PM was washed at least three times by centrifugation and resuspension in 0.1 M $NaHCO_3$ (pH 8.5) and left overnight in this solution at 4°C (15) before three additional washes in 150 mM KCl. Under the labeling conditions applied (i.e., pH 8.1–8.5, molar fluorescein/BR ratio of 100, 4–6 hr of incubation at 22°C), 0.7–1.0 fluorescein molecule was bound per BR. The time course of pH changes in the aqueous bulk phase was monitored with the pH indicator dye pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate, laser grade, Kodak; Fig. 1; refs. 16 and 17) by laser flash photometry (18, 19).

RESULTS

Covalent Labeling of BR with the pH Indicator Fluorescein.

A pH indicator residing in the aqueous bulk phase monitors H^+ transfer from the membrane surface into the medium but not the appearance of H^+ at the ejection site of the protein (3, 16). We have covalently attached various optical pH indicators to the extracellular surface of BR. Data will be discussed only for two fluorescein derivatives bearing either an isothiocyanate (FITC) or succinimidyl ester (FSE) (F in Fig. 1) linker. Both of these reactive groups conjugate fluorescein covalently with the ϵ -amino group of lysines and with the α -amino group of the N-terminal amino acid (20), which, however, is blocked in mature BR. No differences were observed between these two fluorescein derivatives when conjugated to BR. The carboxamide formed by FSE is more stable than the thiourea formed by FITC (20). On the other hand, if attached to Celite, FITC can be added directly to an aqueous PM suspension, avoiding the use of an organic solvent such as *N,N*-dimethylformamide. However, even at much higher concentrations than applied (4.3%), dimethylformamide does not affect the spectroscopic and functional properties of BR. Covalent binding of fluorescein to BR was verified by PAGE of labeled PM solubilized by SDS. Bacteriorhodopsin as well as various peptide fragments obtained by

FIG. 1. Sketch of BR in the PM with fluorescein (F) covalently bound to Lys-129 via a carboxamide linkage. Pyranine (Py) resides in the aqueous bulk phase.

site-specific proteolysis with chymotrypsin, papain, or proteinase K were highly fluorescent on the gel. From the previously determined amino acid sequence of these fluorescent peptide fragments (21, 22), the fluorescein conjugation site(s) in BR could be unambiguously identified. For example, the chymotryptic C2 fragment (amino acids 1–71, ref. 22) is nonfluorescent in mature BR, excluding labeling of Lys-30, -40, and -41 (and of the α -amino group of the N-terminal amino acid). On the other hand, the C2 fragments with slightly higher molecular weight emerging from two precursor forms of BR (23, 24) are highly fluorescent, indicating labeling of the α -amino group of the N-terminal amino acid in their presequences. Combination of these data with those of papain and proteinase K digestion (21) clearly shows that under labeling conditions yielding 0.7–1.0 bound fluorescein molecule per BR, the dye is specifically conjugated with Lys-129 of the mature BR. In addition, the α -amino group of the N-terminal amino acid in all of the 3–15% (depending on the cell growth conditions) precursor forms of BR in the PM is labeled. Both of these conjugation sites are at the extracellular side of the PM (Fig. 1) and freely exposed at the BR surface (4, 21). It has been reported that biotin *N*-hydroxy-succinimide ester labels (under conditions very similar to those applied here) solely an extracellular lysine residue (15). Our control experiments demonstrated that neither the labeling procedure nor up to two fluoresceins bound per BR influenced the photocycle kinetics of the rate and the stoichiometry of H^+ transport. This is in accordance with previous observations that biochemical modifications of lysines do not alter the functional properties of BR (25, 26). When living halobacteria devoid of the cell wall were incubated with FITC/Celite, the same labeling pattern and light-triggered H^+ -transfer kinetics of the PM were observed (data not shown). This confirms the location of the pH probe at the extracellular surface of BR. The pK_a ranges from 7.6 at 1 mM KCl to 6.6 at 150 mM KCl, proving that fluorescein resides in the diffuse double layer of the PM surface.

Light-Triggered Absorbance Changes of Fluorescein. Light excitation of BR triggers H^+ release into the extracellular medium and subsequent reuptake from the cytoplasm (7, 8). These events were monitored by fluorescein covalently bound to BR. The lower trace in Fig. 2A shows on a

FIG. 2. (A) Time course of light-induced absorbance changes (at 489 nm) of native BR (upper trace) and fluorescein-labeled BR (lower trace); pH 7.5; OD_{BR} 0.9; optical pathlength, 1 cm. (B) Effect of increasing buffer concentrations. Traces: a, no Tris (difference between the two traces in A); b, 0.2 mM; c, 0.5 mM; d, 1 mM; e, 100 mM Tris.

logarithmic time scale the absorbance changes at 489 nm, the absorbance maximum of fluorescein. The signal is a superposition of absorbance changes of the BR photocycle (upper trace) and of fluorescein. The response of fluorescein (Fig. 2B, trace a) is obtained by subtracting the absorbance changes of labeled and unlabeled PM (Fig. 2A) measured under identical conditions. Even at the relatively low labeling extent of 0.7 fluorescein per BR, the fluorescein response contributes to a 50% increase in the absorbance changes, thus excluding artifacts by subtraction of the two data traces.

The fluorescein response at 489 nm (Fig. 2B, trace a) is characterized by a fast absorbance decrease followed by a multiphasic relaxation to the baseline. Control experiments with fluorescein dissolved in water proved that the signal was not caused by light excitation of the dye. To establish the pH-sensitive component of the fluorescein response, measurements were performed in the presence of buffers (Tris, imidazole). These buffers did not affect the photocycle kinetics. Increasing the buffer concentration led to a pronounced decrease in amplitude (Fig. 2B, traces b–d). However, even at a very high buffer concentration, a signal component remained (trace e). By choosing appropriate sample conditions, the complex absorbance changes of fluorescein-labeled PM can be evaluated. As described above, subtraction of the absorbance changes from labeled and

unlabeled BR, both measured in the absence of buffer, results in the pure fluorescein response (Fig. 2B, trace a); the pH-sensitive component of the fluorescein response, reflecting changes in surface pH is obtained by comparing labeled BR in the absence and presence of buffer; the pH-insensitive component of the fluorescein response, reflecting alterations in surface potential (see below) evolves from the comparison of labeled and unlabeled BR in a strongly buffered medium.

H⁺ Migration. The difference between subsequent measurements of fluorescein-labeled PM without buffer and the same PM in a highly buffered solution is depicted in Fig. 3 (trace F). The decrease in absorbance corresponds to a transient acidification of the environment of the surface-bound fluorescein, reflecting H⁺ release to the extracellular surface of BR. The time constant for the protonation of fluorescein is $\tau_1 = 63 \pm 8 \mu\text{s}$ at 22°C. (Values reported are means and SD of determinations carried out on three samples.) Deprotonation of fluorescein, reflecting reestablishment of the original pH, proceeds in two steps ($\tau_2 = 775 \pm 152 \mu\text{s}$; $\tau_3 = 8.9 \pm 3.8 \text{ ms}$).

Trace P in Fig. 3 represents the absorbance changes of the highly water-soluble pH indicator pyranine. The response of pyranine to the H⁺-pumping activity of BR is identical with that of the water-soluble fluorescein analogue 5(6)-carboxyfluorescein. The absorption decrease of pyranine is due to H⁺ release from BR into the aqueous bulk phase. The time constant of $630 \pm 188 \mu\text{s}$ is about 1 order of magnitude larger than the time constant for the protonation of surface-bound fluorescein. The protonation of pyranine is rather congruent with the first deprotonation reaction of the surface-bound fluorescein (775 μs). The pH of the aqueous bulk phase is subsequently raised to the initial value due to the H⁺ reuptake by BR. Pyranine monitors this process with almost the same time constant ($\tau_2 = 12.1 \pm 2.5 \text{ ms}$) as the second deprotonation reaction of fluorescein (8.9 ms).

In Fig. 3, the kinetics of the M intermediate are compared with the pH changes monitored by both pH indicators. Rise and decay of M exhibit a complex behavior. Under the

FIG. 3. Comparison of the time course of pH changes with rise and decay of the M intermediate. M (14 μM BR) was monitored at 412 nm while BR-bound fluorescein (F, 13 μM) and pyranine in the aqueous bulk phase (P, 40 μM) were measured at 489 nm and 457 nm, respectively. Temperature was 22°C; pH 7.5; 150 mM KCl; due to a pK of about 7.2 and suitable optical properties, pyranine was used in the same sample together with fluorescein. Time constants (numbers in brackets represent amplitudes in percent of the total amplitude): $\tau_1^M = 1.4 \mu\text{s}$ (−8%), $\tau_2^M = 44 \mu\text{s}$ (−35%), $\tau_3^M = 149 \mu\text{s}$ (−57%), $\tau_4^M = 1.4 \text{ ms}$ (32%), $\tau_5^M = 4.7 \text{ ms}$ (59%), $\tau_6^M = 14 \text{ ms}$ (9%), $\tau_1^F = 63 \mu\text{s}$ (100%), $\tau_2^F = 775 \mu\text{s}$ (−32%), $\tau_3^F = 8.9 \text{ ms}$ (−68%); $\tau_1^P = 630 \mu\text{s}$ (100%), $\tau_2^P = 12.1 \text{ ms}$ (−100%).

applied conditions, six exponentials are required to fit the data satisfactorily. The time constants are listed in the legend of Fig. 3. To judge the kinetic correlation between the photocycle and the H⁺-transfer steps monitored with the pH indicators, as well as to determine the activation energies of the respective reactions, temperature dependencies were investigated in the range 10–50°C. Fig. 4A is an Arrhenius plot comprising the rise of M, the protonation reactions of surface-bound fluorescein and of pyranine in the water phase, and the first deprotonation reaction of fluorescein. The rise of M was fitted with three exponentials. With the time resolution of the experimental setup (50 ns), the exponential with the smallest time constant could be resolved only up to 30°C.

The protonation reaction of surface-bound fluorescein [$E_a(\tau_1^F) = 35.0 \text{ kJ/mol}$] requires only half of the activation energy required by the two major components of the M rise [$E_a(\tau_2^M) = 63.8 \text{ kJ/mol}$; $E_a(\tau_3^M) = 63.7 \text{ kJ/mol}$]. As a consequence, the H⁺ release time monitored by fluorescein matches at elevated temperatures the time for slow M rise (τ_3^M), whereas at lower temperatures it fits to the fast M rise time (τ_2^M). Measurements at temperatures below 10°C show that the protonation reaction of fluorescein is equal to τ_2^M (19). Comparing the Arrhenius plot of the protonation reaction of pyranine (τ_1^P) with that of the first deprotonation step of fluorescein (τ_2^F) demonstrates that over the entire temper-

FIG. 4. Arrhenius plot of the M intermediate and the pH indicators fluorescein and pyranine. Time constants are the mean of three independent measurements. (A) *, τ_1^M , first phase of M rise ($E_a = 68.0 \text{ kJ/mol}$); Δ , τ_2^M , second phase of M rise ($E_a = 63.8 \text{ kJ/mol}$); $\blacktriangle$, τ_3^M , third phase of M rise ($E_a = 63.7 \text{ kJ/mol}$); $\circ$, τ_1^F , protonation of fluorescein ($E_a = 35.0 \text{ kJ/mol}$); $\bullet$, τ_2^F , first phase of fluorescein deprotonation ($E_a = 38.8 \text{ kJ/mol}$); $\square$, τ_1^P , protonation of pyranine ($E_a = 41.1 \text{ kJ/mol}$). (B) *, τ_4^M , first phase of M decay ($E_a = 76.7 \text{ kJ/mol}$); Δ , τ_5^M , second phase of M decay ($E_a = 68.6 \text{ kJ/mol}$); $\blacktriangle$, τ_6^M , third phase of M decay ($E_a = 50.6 \text{ kJ/mol}$); $\circ$, τ_2^F , second phase of fluorescein deprotonation ($E_a = 45.1 \text{ kJ/mol}$); $\square$, τ_2^P , deprotonation of pyranine ($E_a = 58.8 \text{ kJ/mol}$).

Fig. 5. Light-induced transient surface potential changes of PM. Lower trace, fluorescein covalently linked to BR ($\lambda = 489$ nm, $\tau_1 = 4.4$ ms, $\tau_2 = 11.8$ ms). Upper trace, di-4-ANEPPS ($\lambda = 420$ nm, $\tau_1 = 2.9$ ms, $\tau_2 = 9.9$ ms). Temperature was 22°C, pH 7.5, 100 mM Tris, 150 mM KCl. (Inset) Structure of di-4-ANEPPS.

ature range investigated, the time constants of the two reactions are the same within the experimental error. Fig. 4B illustrates the kinetics after the M intermediate reached its maximum concentration. Clearly, the two exponentials of the M decay with the largest amplitudes (τ_4^M , τ_5^M) are not related to the deprotonation reaction of the two pH indicators (τ_2^P , τ_3^F). Rather, the H^+ reuptake monitored by both fluorescein and pyranine is coupled to the slowest relaxation time (τ_6^M), which contributes at this pH only little to the M decay.

The time for the appearance of the H^+ at the surface, as reflected by the protonation of fluorescein, is less affected by increasing buffer concentrations, whereas the subsequent H^+ transfer from the surface into the bulk water phase is strongly accelerated (Fig. 2B). The protonation reaction of pyranine exhibits the same buffer dependence (data not shown; compare ref. 16) as the first phase of the deprotonation reaction of fluorescein.

Transient Surface Potential Changes. When labeled PM is measured in a highly buffered suspension (Fig. 2B, trace e), a pH-independent optical signal of surface-bound fluorescein is observable (Fig. 5, lower trace). This signal is supposed to reflect the kinetics of transient surface potential changes. To verify this assumption we applied the potentiometric dye di-4-ANEPPS. This styryl dye intercalates with its two *n*-butyl chains into the lipid domains (27). As shown in Fig. 5, the optical signals of di-4-ANEPPS (upper trace) and fluorescein (lower trace) exhibit the same time dependence. This indicates that the pH-independent response of the surface-bound "pH"-probe fluorescein monitors a transient change in surface potential. It occurs with $\tau = 3$ ms and decays with $\tau = 10$ ms at 22°C (di-4-ANEPPS).

DISCUSSION

H^+ Transfer. Triggering the BR photocycle by a laser flash results in light-induced H^+ release from BR. The kinetics of the pH indicator response strongly depend on the location of the probe. Fluorescein covalently attached to Lys-129 at the extracellular surface of BR monitors the released H^+ about 10 times faster than pyranine residing in the aqueous bulk phase distant from the PM (Figs. 1, 3, and 4). The protonation reaction of fluorescein has a rate constant similar to the electrical B2 component measured by Liu (28). We conclude that the B2 component reveals the appearance of H^+ at the

surface and not the release of H^+ into the aqueous bulk phase.

Fluorescein protonation is followed by a deprotonation step that occurs concurrently with the protonation of pyranine. After the H^+ is pumped from the BR interior to the extracellular surface of the PM, it dwells at the membrane surface for about 700 μ s. This indicates a diffusion barrier for H^+ at the PM surface. Subsequently, this H^+ excess dissipates into the bulk. Both the first deprotonation step of fluorescein and the protonation of pyranine reflect this process (Fig. 3), as confirmed by measurements at various temperatures (10–50°C, Fig. 4A). Therefore, pH indicators not bound to BR are inadequate to measure the kinetics of H^+ ejection by BR. Their response is delayed because of diffusion controlled transfer of H^+ from the surface into the aqueous bulk. The surface-bulk transfer has no optical counterpart in the photocycle (Fig. 4A). However, in electrical measurements a component in the same time domain had often been observed (29).

As compared to the velocity of H^+ in pure water, the apparent rate of dissociation of H^+ from the membrane surface into the bulk is slowed in the presence of buffering surface groups (30). Addition of mobile buffers increases the clearance of H^+ from the surface by collisional H^+ transfer (2, 16). By increasing the concentration of mobile buffer, surface-bulk transfer is accelerated while the rate of appearance of the pumped H^+ at the surface is hardly influenced (Fig. 2B). Thus, the dwell time for H^+ at the membrane surface is reduced. To quantify the response, the absorbance changes of the pH indicators were titrated. We confirmed earlier measurements where the stoichiometry of pumped H^+ per cycling BR is about 1 when detected with a pH indicator that resides in the bulk water phase (16, 31). However, from the transient amplitude of the protonation of surface-bound fluorescein, an *apparent* stoichiometry between 3 and 4 is calculated. This high value is readily explained by considering the dwell time of H^+ at the surface. The reaction volume is smaller than the volume of the bulk water phase. Thus, the H^+ -pumping activity of BR transiently lowers the pH at the surface to a larger degree than in the bulk.

In Fig. 3, the kinetics of the M intermediate are compared with the signals arising from both indicators. Fitting the data trace measured at 412 nm (i.e., at the maximum of M absorbance change) required six exponentials (legend of Fig. 3), reflecting the K–L, L–M, M–L, M–N, N–M, and N–O transitions (32). At 22°C (Fig. 3) the protonation of fluorescein proceeds with a slightly slower rate than the second component of the M rise (τ_2^M). At 10°C (Fig. 4A) and at even lower temperatures (down to –15°C; ref. 19) the two rates are almost identical. This demonstrates that the pumped H^+ appears at the extracellular surface during the L–M transition, concomitantly with the transfer of the Schiff base H^+ to Asp-85 (9, 10). Thus, the apparent diffusion coefficient of the H^+ from the Schiff base to the extracellular surface of BR (distance, ≈ 20 Å; ref. 6) is $D_{app} = 3.2 \times 10^{-10}$ cm²/s (calculated from the Einstein–Smoluchowski relation $x^2 = 2Dt$, with $\tau = 63$ μ s for the protonation reaction of fluorescein). This value represents a lower limit for the apparent H^+ diffusion within BR. Remarkably, it is comparable to the apparent coefficient of diffusion of H^+ across pure lipid bilayers (1, 33). At higher temperatures the rate of fluorescein protonation approaches that of the M₃ component (Fig. 4A). This is due to the temperature dependence of H^+ diffusion to the surface-bound fluorescein, which has a lower activation energy than the activation energies for the M rise processes. The value of 35 kJ/mol for the protonation reaction of fluorescein indicates that it does not occur by pure diffusion of H^+ in water ($E_a = 18$ kJ/mol; ref. 34) but involves various protonation/deprotonation reactions with amino acid side chains (2).

The reuptake of a H^+ by BR coincides with the slowest component of the M decay (Figs. 3 and 4B). This component, attributed to the N–O transition (18, 32), has only a small amplitude at pH = 7.5 and $\lambda = 412$ nm. By increasing the pH, the amplitude also increases while the rate of the N decay decelerates. The rate of H^+ reuptake measured by pH indicators with higher pK values exhibits the same pH dependence (data not shown). This supports the suggestion that H^+ reuptake by BR accompanies the N–O transition.

The lateral distance between H^+ ejection site and pH indicator (fluorescein bound to Lys-129) does not affect the measured H^+ -release kinetics. The apparent diffusion coefficient of protons for a 14 μ M PM suspension with the buffering capacity $\beta_{PM} = 128 \mu\text{M}/\text{pH}$ is $D_{app} = 3.4 \times 10^{-7} \text{ cm}^2/\text{s}$ at pH = 7.5 (from equation 19 in ref. 30). With $x^2 = 2Dt$ and $x = 3$ nm for the diameter of the BR surface, the time for H^+ diffusion is only 134 ns. This is much faster than the time constant for the protonation reaction of fluorescein (63 μ s). Assuming free H^+ diffusion as in bulk water ($D_{H^+} = 9 \times 10^{-5} \text{ cm}^2/\text{s}$; ref. 2), the diffusion time would be 500 ps!

Transient Surface Potential Changes. Fluorescein covalently attached to BR exhibits absorbance changes in the millisecond time domain (Fig. 5) that are still present at high Tris concentration and are also observable with more hydrophobic buffers such as imidazole. These pH-independent absorbance changes are attributed to a transient change in surface potential caused, e.g., by alterations in the tertiary structure of BR (10, 13, 14). Thus, fluorescein, which is located in the diffuse double layer, might enter another environment. A steep gradient in polarity exists at the interface from the almost nonpolar hydrocarbon region ($\epsilon = 2$) via the surface ($\epsilon = 20$ –35) to the polar bulk water phase ($\epsilon = 78$) (33, 35). The decrease in fluorescein absorbance demonstrates that its pK is transiently shifted toward a higher value. Only a more nonpolar environment and/or an additional negative charge (due to deprotonation of an amino acid such as Asp-96) can account for this effect. The potentiometric dye di-4-ANEPPS, which is solely sensitive to changes in the surrounding electrical field (36), undergoes absorbance changes with a time course comparable to that of surface-bound fluorescein (Fig. 5). Such congruent behavior of surface-bound fluorescein and di-4-ANEPPS was also reported in experiments with a Na^+/K^+ -ATPase (37).

Conflicting results concerning the correlation of light-induced surface potential changes and of the photocycle have been published (38, 39). Our investigation shows that the modulation of the surface potential occurs within the time domain of the M–N transition. Further, the M–N transition (τ_4^M in Fig. 3) is pH-independent, and the same pH independence is observed for the rise of the potential signal. The relaxation of this change in surface potential correlates with the N–O transition. This is clearly visible at alkaline pH values, where the N–O transition is prominent in the M decay via the M–N back reaction (data not shown).

The availability of pH- and potential-sensitive probes located at known sites of the membrane surface will further facilitate the elucidation of the BR pumping mechanism and can contribute to the understanding of energy conversion by biological membranes.

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