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pH dependence of light-induced proton release by bacteriorhodopsin

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We have measured the current generated by light-activated proton release from bacteriorhodopsin into solution as a function of both pH and ionic strength. We find that proton release into solution decreases with increasing pH with an intrinsic pK_a of 8.2 ± 0.2. This pH dependence indicates that the deprotonation of a certain group inhibits or abolishes proton release. Under physiological conditions, this group either releases a proton directly into solution or interacts with the site of proton release The most immediate candidates for this protonatable species are tyrosine-57, tyrosine-185, arginine-82, and water; acting individually or cooperatively. The salt dependence of the apparent pK_a of this group also allows us to calculate the surface charge density of about −5 charges per bacteriorhodopsin, compatible with previous estimates.

Purple membrane, Proton pump

1. INTRODUCTION

Bacteriorhodopsin is a light-activated proton pump found in the purple membrane of the archaebacterium *Halobacterium salinarium*. Its purple color is due to a retinal moiety bound to the ε amino group of an internal lysine via a protonated Schiff base. The mechanism and pathway of the proton release process are not well understood. Upon illumination, bacteriorhodopsin undergoes a photoreaction which can be presented as a cycle consisting of discrete spectral intermediates; proton release occurs during the transition between the L and M intermediates (Fig. 1) [1]. Recent evidence suggests that there must be at least two sequential steps in the release of the proton into solution (recently reviewed by Ebrey [1]). The first proton transfer occurs on the same timescale as the transition from the L intermediate to the M intermediate during which the Schiff base deprotonates and Asp-85 protonates. Without adding a significant time delay, a second transfer occurs from another group near Asp-85 to the external solution since protons have been detected in the external medium during this timescale [2,3] yet Asp-85 remains protonated until the O intermediate [4–6].

This proton release into solution can be detected in photocurrent measurements as an exponentially decaying 85 μs component (the B2 component) [3,7,8]. The integrated area of the B2 component is proportional to the number of protons released into solution. Increasing the pH results in a decrease in the area of the B2 component. Concurrently, two faster photocurrent components with time constants of 0.3 and 6 μs appear [9]. Due to their insensitivity to the conditions of the external bathing media other than pH, these faster current components have been attributed to nonpumping internal charge movements [9]. Here we have studied the effect of ionic strength on the pH titration of the B2 photocurrent, associated with proton release, to determine the intrinsic pK of the deprotonation event and also the surface charge density of the membrane.

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2. EXPERIMENTAL

Bacteriorhodopsin was prepared as membrane sheets (purple membrane) using the method of Becher and Cassim [10] but omitting DNase treatment Purple membrane was oriented and immobilized in a polyacrylamide gel using the method of Dér et al. [11] as modified by us [9]. The experimental setup for preparing the oriented samples and measuring their photocurrents have been previously given [9,12]. Prior to the measurements, the gel pieces were stirred in a solution of the desired ionic strengh overnight or longer during which the bathing solution was changed at least four times. Individual gel pieces were then placed in 15 ml of the desired KCl solution, and the pH adjusted by addition of KOH. After overnight incubation of the sample, photocurrents were recorded. The pH of the bulk solution was measured at the time of the photocurrent measurement.

Areas of the B2 component were determined by curve-fitting 500 μs current traces to a single exponential and a baseline. The area of the B2 component reflects the number of charges (protons) that move in this timescale (proton release) [7,9] and was determined by the product of the amplitude and lifetime of the exponential B2 decay. These areas were then normalized relative to the amount of M intermediate formed as monitored spectroscopically at 410 nm.

From the salt-dependence of the titration curves, calculations of intrinsic pK_a values and surface charge density determination were performed using the Gouy–Chapman equation as described by Koutalos et al. [13].

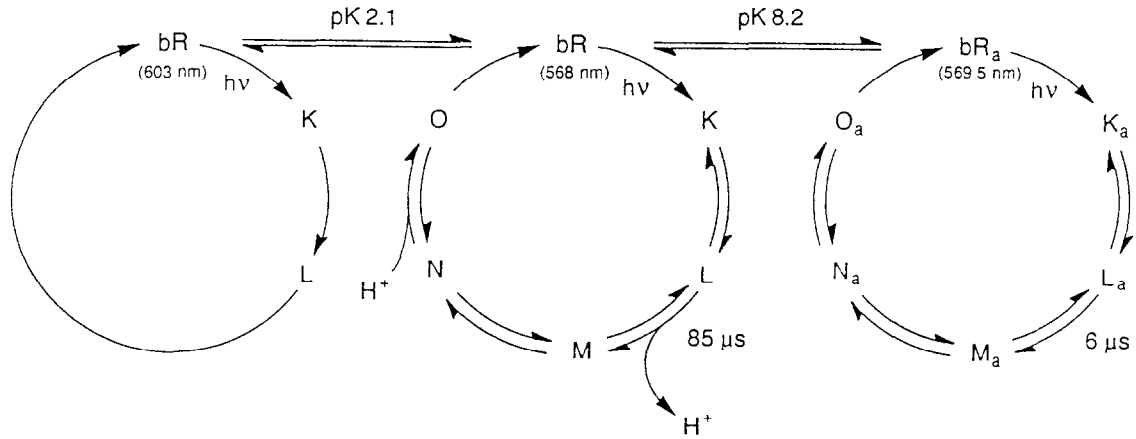

Fig. 1. Model for bacteriorhodopsin photocycle at different pH values. Depending on the pH, there is a different proportion of the pH-dependent forms of bacteriorhodopsin: bR_{605} (blue form), bR_{568} (normal form), and $bR_{569.5}$ (alkaline form) Each of these species is able to undergo its own photocycle.

3. RESULTS

Fig. 2A contains representative photocurrent traces at different pH values. As pH increases, the 85 μs component decreases and two fast components appear as described by Liu [9]. Fig. 2B shows the relative area of this 85 μs photocurrent component as a function of pH at KCl concentrations ranging from 50 mM to 500 mM and the nonlinear least squares fit of the data to the Henderson-Hasselbach equation. The area decreases sigmoidally with increasing pH in agreement with the results of Liu [9]. The salt-dependence of this transition is clear from Fig. 2B. As ionic strengh increases, the apparent pK_a for the transition decreases. This behavior is indicative of a process modulated by the large negative surface potential of the membrane and described by Gouy–Chapman theory. The net negative charge on the surface of the membrane reduces the pH at the surface of the protein relative to that of the bulk solution. Increasing the ionic concentration masks the negative surface charges allowing for the proton concentration in the bulk solution to approach that at the surface. Thus at infinite ionic strength, the surface and bulk pH would be the same, and the measured pK_a of the transition would be the real pK_a (referred to as intrinsic pK).

Analysis of this data following the reformulation of the Gouy–Chapman equation by Koutalos et al. enables the determination of both the intrinsic pK_a and surface charge density [13]. We find the intrinsic pK_a for the transition to be 8.2 ± 0.2. The surface charge density is calculated to be about −5 charges per 1140 Å^2, the area of the membrane per pigment molecule.

4. DISCUSSION

The simplest interpretation of our results is that at high pH a single group deprotonates and that this event decreases light-induced proton release: either directly because the group no longer has a proton to release or indirectly because the increase in negative charge in the active site alters the release process. Tyr-57, Tyr-185, and Arg-82 (and possibly a bound water molecule [14–16] or an aspartate residue [17]) are likely candidates for this titratable residue because of their proximity to the active site of proton release (extracellular half of the membrane) [18] and their ionizability.

At high pH, a tyrosine in bacteriorhodopsin has been proposed to be able to deprotonate with an intrinsic pK of about 8.3 resulting in a red-shifted form of bR ($bR_{alkaline}$), which undergoes a photocycle different from that at neutral pH (Fig. 1) [19]. Govindjee et al. [20] report spectral titrations at 240 nm showing that this low pK_a tyrosine a bacteriorhodopsin is missing in a mutant in which Tyr-57 is replaced with Asn, suggesting that this is the low pK_a tyrosine. Here, we observe a decrease in light-induced proton release with increasing pH with an intrinsic pK_a similar to the reported tyrosine pK_a, suggesting that normal proton release and the slow forming (85 μs) M intermediate are associated with the protonated state of a tyrosine. A deprotonated tyrosine in the proton release active site results in an extra negative charge added to the cluster of residues comprising the counterion to the Schiff base and likely changes the normal interactions between them. Substitutions of either Tyr-57 with Asn [20,21] or Tyr-185 or Tyr-57 with Phe [23,23] have been shown to alter normal photocycling and proton pumping.

The role of Arg-82 is intriguing because substitution of this positively charged residue with a neutral one (Ala) makes the charge in the active site more negative and accelerates the formation rate of the M intermediate similar to that of the alkaline form of the native bacteriorhodopsin (which we believe is attributed to tyrosine deprotonation [19]) [24–26]. Lin et al. [25] have discounted the role of Arg-82 in the alkaline form of bacteriorhodopsin because the decay rate of the M in-

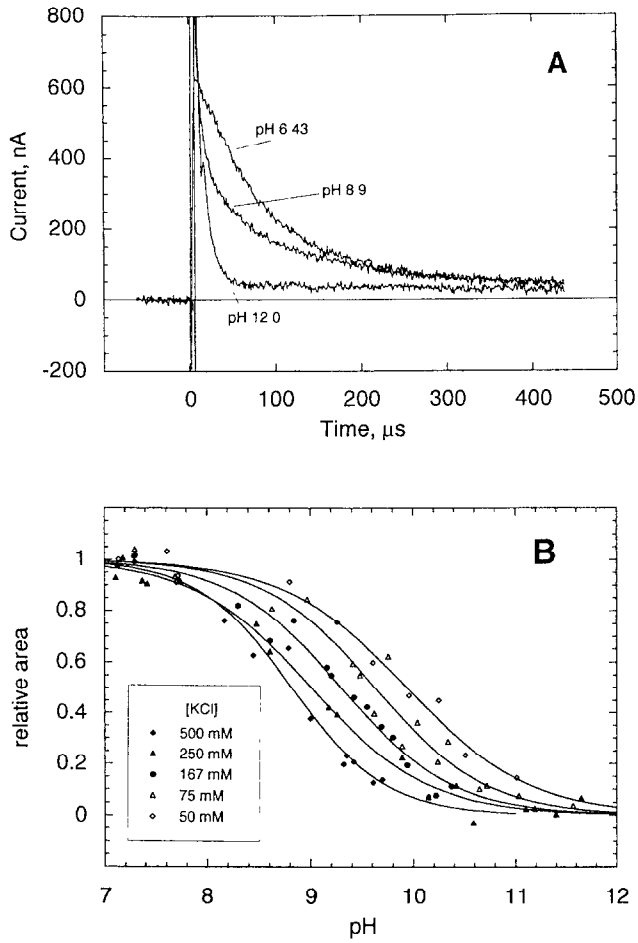

Fig. 2. (A) Representative photocurrent traces illustrating the pH dependence of the B2 component (85 μs component) photocurrent signal. These samples are in 100 mM KCl. (B) Relative areas of the B2 component at various pH values and ionic strengths. The curves represent fits of the titration data to the Henderson–Hasselbach equation ($n = 0.85 \pm 0.1$).

termediate of the Arg-82 Asn mutant was much faster than that of the alkaline form; however, the decay rate is more likely controlled by the protonation state of Asp-96 located on the cytoplasmic half of the protein, while the formation rate of the M intermediates is regulated by changes on the extracellular half of the protein. Zimányi et al. [16] discuss the need for an unknown proton releasing group with an apparent pK_a of 5.8 during the photocycle (called 'X' in their paper). They, however, discount Arg-82 as their X because an apparent pK_a of 5.8 during the photocycle is too low for arginines and instead suggest a water bound to Arg-82 [16]. We propose a group with an intrinsic pK_a of 8.2 in the ground state (closer to 9.3 under Zimányi et al.'s ionic conditions) as a possible proton source. Such an alkaline pK_a, makes Arg-82 a very plausible candidate for X. During the transition from the L to M intermediate, the environment around the Schiff base changes greatly; the pK_a of the Schiff base, for example, has a pK_a shift of more than 7 pH units. If our measured protonatable group is the same as that of Zimányi et al., then this x would have a pK_a shift of about 3 pH units from about 9 to 6 (using Zimányi et al.'s ionic conditions), which although large could reflect the same environmental changes seen by the Schiff base.

Lin et al. [25] also report the loss of transient absorbance changes in the near-uv spectrum for their Arg-82 mutant. This result suggests an interaction between Arg-82 and tyrosine(s) during the L to M intermediate transition, consistent with our results here and our studies on Arg-82 and Tyr-57 mutants and spectral titrations [19,20,26]. Under alkaline conditions, a negatively charged tyrosinate near Arg-82 (e.g. Tyr-57 or Tyr-185) would neutralize the charge on Arg-82, resulting in an environment similar to that in the Arg-82 mutant.

Our salt-dependent pK_a measurements have also yielded an estimate of the surface charge density of −5 charges per bacteriorhodopsin (presumably on the N-terminal side of the membrane). The value we calculate is somewhat larger than the generally accepted value of about 2 negative charges per bacteriorhodopsin [27]. However, because the surface charge density is determined at alkaline pH conditions, additional residues (at least one tyrosine, perhaps) are deprotonated making the surface more negative. Jonas et al. [27] have summarized surface charge densities of bacteriorhodopsin reported as well as inferred from salt-dependent data. In this review, it appears that those values derived from salt-dependent photocycle measurements are slightly more negative (−3 to −6 charges per bacteriorhodopsin) than those from salt-dependent properties of the ground state (−2 to −5 charges per bacteriorhodopsin). Our value seems reasonable considering those previously reported.

Our photocurrent measurements demonstrate the significance of changes in protonation state of a specific group in regulating protein surface and function. At high pH, a group in bacteriorhodopsin deprotonates, resulting in a more negative active site. This change inhibits or abolishes normal proton release and accelerates formation of the M intermediate. The intrinsic pK_a that we determined for the loss of normal proton release correlates with the previously found titration of a tyrosine residue [19]. The removal of the positively charged Arg-82 results in behavior similar to that of the alkaline form [26]. We see either tyrosinate formation or deprotonation of Arg-82 itself as neutralizing the positive charge of Arg-82 and preventing normal proton release.

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