# APPARENT pK OF PHOTOLABILE PROTON BINDING TO BACTERIORHODOPSIN

Robert Renthal

Division of Earth and Physical Sciences, University of Texas at San Antonio, and Department of Biochemistry, University of Texas Health Science Center at San Antonio
San Antonio, Texas 78285

Received May 16,1977

SUMMARY: Low intensity steady illumination induces release of protons from isolated sheets of purple membrane at alkaline pH. This effect was studied by differential titration under various conditions of ionic strength and detergent. The stoichiometry of steady-state proton release is consistent with a three-state model for a single proton binding site. The apparent pK of the photodissociable proton binding is 9.9 in water.

A simple light-activated proton pump occurs in the purple membrane, a differentiated, crystalline membrane region of the extremely salt-dependent bacteria Halobacterium halobium (1). The photoreceptor of the purple membrane is bacteriorhodopsin, the only protein constituent, which contains the prosthetic group vitamin A aldehyde linked as a Schiff's base. The chemical mechanism of light-induced proton pumping is not yet known. Both the retinal chromophore of bacteriorhodopsin(2) and the protein itself (3) undergo changes upon illumination. As part of an investigation of amino acid side chain involvement in the proton pump, I have studied the photolabile proton binding to bacteriorhodopsin at alkaline pH by differential titration. The results suggest that a single group with a pK of about 10 could account for the proton release site of the pump.

## MATERIALS AND METHODS

Purple membrane was isolated from Halobacterium halobium $S_9$, (provided by Dr. J. Lanyi, Ames Research Center, Cal. 94035) by a procedure similar to that reported by Oesterhelt and Stoeckenius(4). Triton X-100 was obtained from Sigma.

Measurements of pH changes were made by a similar technique to that described previously (5). Each experiment was a combined titration and





*ISSN 0006-291X*

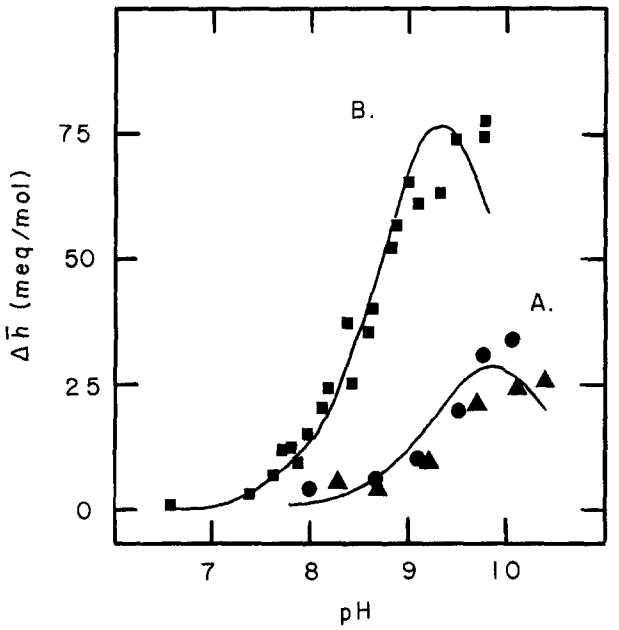


Figure 1. Measured steady-state proton release from purple membrane under continuous low intensity illumination. Lines are calculated from equation (1) using the constants given in Table I. Conditions: A. circles: water; triangles: 0.015 M NaCl. B. 3 M Na Cl.

differential titration. In most experiments, a stream of nitrogen was passed over the surface of the continuously stirred sample (2.0 ml of 1.0 x $10^{-5}$ M bacteriorhodopsin). However, neither stirring nor air had a significant effect on the results. Titrations with either 0.020 N NaOH or 0.020 N HCl were delivered through a very fine glass capillary from a syringe microburet (Micrometric Instrument Co.). Increments of 1µl were added near neutral pH and 5-10 µl near pH 9 or 6. Approximately every 0.4 pH units, the titration was stopped and the sample was illuminated.

The actinic light source was a Sylvania tungsten halogen projector lamp (ELE quartz, 80 W, 20 V) filtered through 6 cm of 1% $CuSO_4$ and an Oriel G-572 560 nm interference filter. The light was placed 10 cm from the sample. A Tektronix P6058 temperature probe showed less than 0.2° C increase in temperature when illuminated with this light source. The electrode was covered with aluminum foil, and it gave no detectable light response either in buffer or with denatured bacteriorhodopsin. The light-induced pH changes were recorded, usually from four 20 sec illuminations. The titration was then resumed. For most experiments the on and off changes were nearly equal. Thus, eight measurements were averaged to obtain one data point. In some cases, the proton release or uptake contained more than a single kinetic process. In all experiments, only the fastest process was measured.

The light-induced pH changes were converted to changes in proton binding ($\Delta\bar{h}$, in equivalents per mole of bacteriorhodopsin) by calculating the product

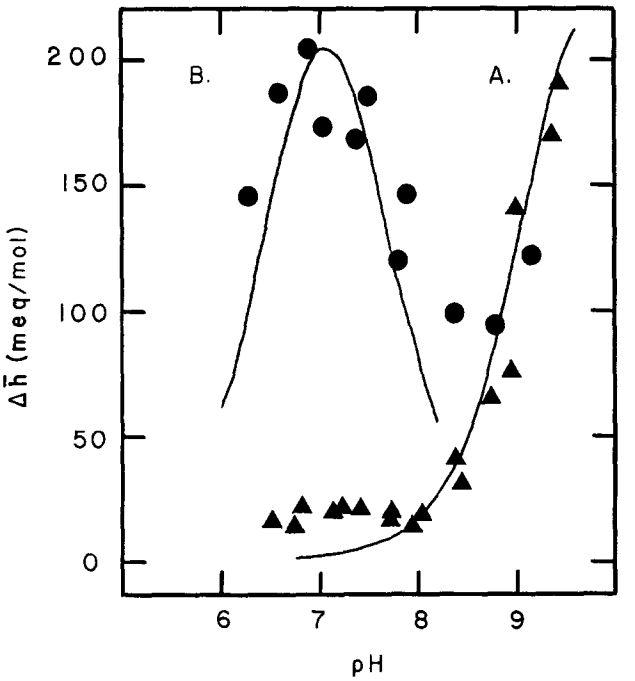


Figure 2. Proton release by continuously illuminated purple membrane in detergent. A. 0.4% Triton X-100. B. 0.1% Triton X-100, 3 M NaCl. Lines are calculated from equation (1) using the constants given in Table I.

of the slope of the titration curve at a particular pH (the buffering capacity, in equivalents per mole of bacteriorhodopsin per pH unit)times the magnitude of the light-induced pH change, ΔpH. The light intensity was measured with a thermopile (Eppley laboratory, Inc.) calibrated from a National Bureau of Standards radiation standard. With identical conditions to those used for the light-induced pH change experiments, the light intensity was measured at $3.46 \times 10^4$ ergs $cm^{-2}$ $sec^{-1}$.

## RESULTS

Isolated sheets of purple membrane suspended in water give only very small changes in pH under steady illumination (figure 1A). The maximum measured ΔpH (at the low light intensity used) was about 0.0002 at pH 7.8. The maximum $\Delta\bar{h}$ measured was 0.03 eq/mole at pH 10.4. The differential titration curve shows increasing proton release with increasing pH up to pH 10. In very dilute NaCl (0.015 M) (figure 1A) $\Delta\bar{h}$ was virtually the same as in distilled water. In concentrated NaCl, over a wide range of pH, the light-induced pH changes were considerably larger than the maximum change observed in water.

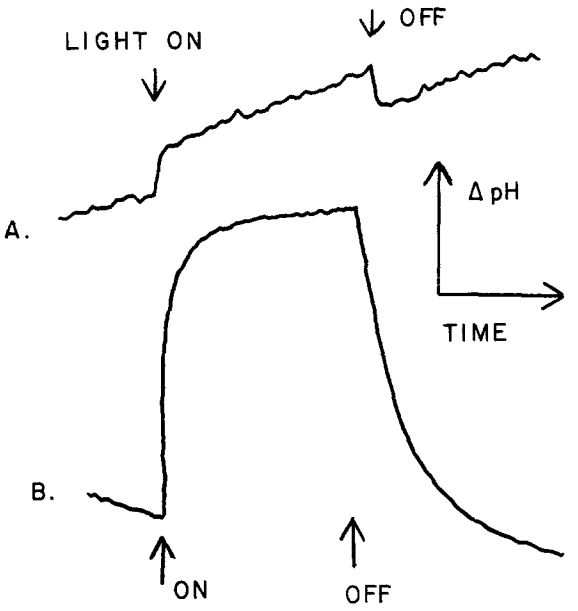


Figure 3. Tracing of recorded pH changes of illuminated purple membrane. A. purple membrane in 0.015 M NaCl (figure 1A) at pH 10.4. B. purple membrane in 0.1% Triton X-100, 3 M NaCl (figure 2B) at pH 7.8. Scale: A. bars represent 0.2 min and -0.001 ΔpH. B. 0.5 min and -0.008 ΔpH.

The maximum ΔpH observed in 3 M NaCl was 0.004 at pH 8.4, and $\Delta\bar{n}$ reached a value of 0.08 eq/mole at pH 9.8 (figure 1B).

Addition of Triton X-100 greatly alters the light-dependent proton binding curves. Under conditions where the membrane is completely dissolved into monomeric protein subunits (0.4% Triton) (6), much larger values of $\Delta\bar{n}$ were found (figure 2A). The protein appears to be irreversibly damaged above pH 9.5. At lower concentrations of detergent, where the membrane is incompletely solubilized, very large light-induced pH changes were observed. In 0.1% Triton and 3M NaCl, a ΔpH of 0.05 was measured at pH 6.9 (figure 2B). This corresponds to a $\Delta\bar{n}$ of 0.205 eq/mole and approaches the steady-state pH gradients maintained by vesicles (7, 5) and whole cells (1,8), or the acidification produced by purple membrane in salt and ether (3).

The present experiments were not designed to measure rates of proton release and uptake. However, the rates of acidification and reprotonation in

0.1% Triton and 3 M NaCl were quite slow, and the recording system probably approximates true rates. The kinetics of proton release and uptake are shown in figure 3. The half time for proton release in 0.1% Triton, 3M NaCl, pH 7.8, was about 2 seconds. The half time for proton uptake was about 6 seconds. There was also a slower, irreversible proton release process above pH 8.

## DISCUSSION

The simplest explanation of the pH dependence of light-induced proton release from the purple membrane under low intensity steady illumination is that there is one photolabile proton binding site per bacteriorhodopsin molecule and three states: I. ground-state bacteriorhodopsin with the photolabile proton bound; II. ground-state bacteriorhodopsin from which the photolabile proton has dissociated; and III. photo-excited bacteriorhodopsin from which the photolabile proton has dissociated. These three states are called RH, R and R*, respectively. In the dark, R and RH are in equilibrium. Under steady illumination, all three states exist in a pseudo-equilibrium:

$$R \underset{}{\overset{K_1}{\rightleftharpoons}} RH \overset{k_1}{\underset{k_2}{\rightleftharpoons}} R^*$$

$K_1$ is the apparent dissociation constant for the photolabile proton binding site. The photoreaction cycle of Lozier, Bogomolni and Stoeckenius (9) is contained within $k_1$ and $k_2$, where $k_1$ is the rate constant for light-induced proton release and $k_2$ is the rate constant for proton uptake. Values for both $k_1$ and $k_2$ have been measured by flash spectroscopy (10). With steady, low intensity illumination, $k_1$ is not constant, but is the quantum yield times the absorbed intensity of incident light divided by the concentration of absorbing species (RH). The light induced change in proton binding, $\Delta\bar{h}$, is the difference between the equivalents of hydrogen ion bound to the photolabile site in the dark per mole of bacteriorhodopsin, $\bar{h}_d$, and the equivalents bound per mole

TABLE I

Constants for equation (1) giving best fit to data.

| Conditions | $k_2$ ($M^{-1}$ $sec^{-1}$) | $K_1$ (M) | Apparent pK |
|---|---|---|---|
| Water | $9.3 \times 10^{9}$ | $1.3 \times 10^{-10}$ | 9.9 |
| 3 M NaCl | $1.0 \times 10^{9}$ | $4.0 \times 10^{-10}$ | 9.4 |
| 0.4% Triton X-100 | $8.0 \times 10^{8}$ | $1.3 \times 10^{-10}$ | 9.9 |
| 0.1% Triton, 3 M NaCl | $2.0 \times 10^{6}$ | $5.6 \times 10^{-8}$ | 7.3 |

$k_1$ was measured as 0.145 $sec^{-1}$ assuming all bacteriorhodopsin is in the form of RH. At RH = 0, $k_1$ = 0.25. $k_2$ in water is from ref. 10.

of bacteriorhodopsin under steady illumination, $\bar{h}_\ell$. Thus, $\Delta\bar{h} = \bar{h}_d - \bar{h}_\ell$. By applying the steady state assumption, the following expression is obtained:

$$\Delta\bar{h} = \left(\frac{H}{K_1 + H}\right)\left(\frac{K_2}{K_1 + K_2 + H}\right) \qquad (1)$$

where $K_2 = k_1/k_2$ and H is the free hydrogen ion concentration. Equation (1) may be fitted to the experimental points using $K_1$ and $K_2$ as adjustable parameters, as shown in Table I.# The data is clearly consistent with a simple three-state model. Moreover, it is possible to obtain the apparent pK of the photolabile proton binding site under various conditions. In water, $K_2$ is known from independent measurements (10). Thus, $K_1$ is uniquely determined to be $1.3 \times 10^{-10}$. This apparent pK of 9.9 suggests side chain ionizations of

# $K_2$ is not a constant, since $k_1$ is a function of pH. However, for the low concentrations of bacteriorhodopsin used, $k_1$ varies over a range of 1.7. This variation has only a small effect, and if included in the calculations would slightly improve the fit.

lysine and tyrosine. The result refers to an "average" binding site and could involve more than one group, as in the case of the Bohr protons of hemoglobin. In terms of this model, the difference between low and high ionic strength in the $\Delta\bar{h}$ vs. pH curves is explained by the well-known effects of ionic strength on pK; and the difference between partly and fully detergent-solubilized membrane suggests a substantial effect (such as a micelle or vesicle permeability barrier) on the rate constants for protonation.

The technique reported here constitutes a simple assay for the activity of the purple membrane proton pump. Actually, the intact membrane is unnecessary for activity studies, since proton release is observed with the monomeric protein in detergent. The model may also be extended to include the light-induced proton uptake displayed by the purple membrane at low pH by introducing a light-induced proton uptake process of low pK.

ACKNOWLEDGMENTS

This work was supported by a grant from Research Corporation. I am grateful to Dr. J. Lanyi for cultures of H. halobium $S_9$, samples of purple membrane, and helpful advice. I thank Drs. J. Zuclich and C. Hiatt for measurement of the actinic light intensity, T. Wright and T. Bennett for dedicated technical assistance, and Drs. P. Hochmann and H. Silber for helpful discussions.

REFERENCES

1. Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. 70, 2853-2857.
2. Lewis, A., Spoonhower, J., Bogomolni, R., Lozier, R., and Stoeckenius, W., (1974) Proc. Natl. Acad. Sci. 71, 4462-4466.
3. Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316-326
4. Oesterhelt, D. and Stoeckenius, W. (1974) Methods in Enzymology, V. 31 (Colowick, S. and Kaplan, N., eds.) pp. 667-678, Academic Press, N. Y.
5. Renthal, R. and Lanyi, J. (1976) Biochemistry 15, 2136-2143
6. Becher, B. and Ebrey, T. (1976) Biochem. Biophys. Res. Commun. 69, 1-6
7. Kanner, B. and Racker, E. (1975) Biochem. Biophys. Res. Commun. 64, 1054-1061
8. Bogomolni, R., Baker, R., Lozier, R. and Stoeckenius, W. (1976) Biochem. Biophys. Acta 440, 68-88
9. Lozier, R., Bogomolni, R. and Stoeckenius, W. (1975) Biophys. J. 15, 955-962
10. Lozier, R., Niederberger, W., Bogomolni, R., Hwang, S-B., and Stoeckenius, W. (1976) Biochem. Biophys. Acta 440, 545-556