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Electric-field-induced Schiff-base deprotonation in D85N mutant bacteriorhodopsin

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ABSTRACT **The application of an external electric field to dry films of Asp-85 → Asn mutant bacteriorhodopsin causes deprotonation of the Schiff base, resulting in a shift of the optical absorption maximum from 600 nm to 400 nm. This is in marked contrast to the case of wild-type bacteriorhodopsin films, in which electric fields produce a red-shifted product whose optical properties are similar to those of the acid-blue form of the protein. This difference is due to the much weaker binding of the Schiff-base proton in the mutant protein, as indicated by its low pK of ≈9, as compared with the value pK ≈ 13 in the wild type. Other bacteriorhodopsins with lowered Schiff-base pK values should also exhibit a field-induced shift in the protonation equilibrium of the Schiff base. We propose mechanisms to account for these observations.**

Bacteriorhodopsin (BR) is a protein found in the cell membrane of the bacterium *Halobacterium salinarium*. BR consists of seven α-helices which span the membrane, forming a channel through which protons can be transported. Upon absorption of a photon, BR undergoes a photocycle which results in the translocation of a proton across the membrane, converting the free energy of the photon into chemical energy for metabolism (1, 2). BR can be isolated from the bacterium in the form of purple membranes (3)—so called because of a strong absorption band centered at 570 nm—in which trimers of BR molecules are incorporated in patches of lipid bilayer. These patches can be assembled into dry films for experimentation (4).

The deep purple color of BR comes from the chromophore retinal, which is bound to a lysine residue inside the membrane channel via a protonated Schiff-base linkage. Unbound retinal in solution is faintly yellow, exhibiting a strong absorption centered at 360 nm. However, upon incorporation into BR, the retinal absorption band center shifts to 570 nm. This *opsin shift* is caused in large part by charge redistribution in the retinal due to the positive charge of the proton at the Schiff base. Deprotonation of the Schiff base during the M intermediate state of the BR photocycle leads to a transient partial abolition of the opsin shift, switching the absorption maximum from 570 nm to 410 nm (1, 2). BR variants in which the lifetime of the deprotonated state is prolonged by chemical treatment or genetic modification are the basis for many proposed technological applications of this protein (5, 6).

In wild-type BR, the Schiff-base proton is strongly bound, as indicated by a pK of ≈13, well outside the range of physiologically important conditions. Application of an external field to films of wild-type BR induces spectroscopic changes that are unrelated to the protonation state of the Schiff base (7, 8). However, there are several mutants and retinal-analog-substituted variants of BR in which the Schiff-base proton is much more weakly bound, as indicated by pK values in the range 7 to 9 (9–12). In one of these variants, harvested from mutant bacteria with the Asp-85 → Asn (D85N) mutation (10), we have discovered that external electric fields cause reversible spectroscopic changes which unambiguously represent Schiff-base deprotonation. Explaining the mechanism of this deprotonation will require the application of recently developed numerical techniques of minimization of the electrostatic energy of the protein and may impose new constraints on its structure (13). In addition, this observation may have great technological significance: it may be possible to exploit the effect, using genetic and/or chemical modification of BR, to produce unique electrochromic materials—"electronic ink"—with important applications in the technology of reflective displays.

EXPERIMENTAL PROCEDURES

Standard procedures with minor variations were used to grow strain S-9 wild-type *H. salinarium* cells and to extract the BR membrane patches (3). Strain L-33 D85N cells were grown in the dark in the presence of Novobiocin, exploiting the fact that the vector that carries the mutated gene also contains the Novobiocin-resistance gene. The isolated membranes were washed repeatedly in deionized water to produce suspensions of typical concentration 6 mg/ml. Titration of a suspension of D85N membrane patches yielded a pK value of 8.9, in agreement with previous measurements (10, 11). Two types of dry film samples were deposited on conductive indium/tin oxide-coated glass substrates. Unoriented samples, in which approximately equal fractions of the membrane patches were aligned with their cytoplasmic sides facing toward and away from the substrate, were prepared simply by drying a drop of BR suspension on the substrate at room humidity overnight. This produced films of moderate optical quality. High-optical-quality, oriented samples, in which a majority of the patches were aligned with their cytoplasmic side facing the substrate, were deposited on the same type of slide by using the technique of electrophoretic sedimentation (4, 7, 8). Optically thick wild-type BR films made in this manner exhibited photovoltages of typically 11.5 V upon illumination by yellow light of intensity 0.15 W/cm^2, indicating a high degree of orientation of the constituent membrane patches. As shown below, the vectorial nature of the electroabsorption spectra of films produced by electrophoresis confirms this result, even in the case of D85N BR films, which produce a very weak dc photovoltage. The D85N films used in these experiments exhibited a peak absorbance of A_{600} = 0.4 to 1.2; assuming an optical density calibration of A_{600} = 0.15 per μm yields film thicknesses in the range 3–8 μm.

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Abbreviations: BR, bacteriorhodopsin; D85N, BR in which Asp-85 is replaced with Asn; D85N/D96N, BR in which Asp-85 and Asp-96 are replaced with Asn; D85N/D212N, BR in which Asp-85 and Asp-212 are replaced with Asn; D85N/D96N/D212N, BR in which Asp-85, Asp-96, and Asp-212 are replaced with Asn.

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For electroabsorption measurements—that is, measurements of electric-field-induced absorption changes—we prepared insulated sandwich samples. Dried films produced as above were spin-coated with a 2-μm-thick film of polyvinyl alcohol and then dried overnight over P_2O_5 (0% relative humidity) or over saturated LiCl (12% relative humidity). They were then covered with a 12-μm-thick Mylar sheet and clamped against a second indium/tin oxide-covered glass slide. This insulation technique allowed us to apply voltages of up to 4000 V (electric field $\approx 6.7 \times 10^5$ V/cm, assuming a sample thickness of 20 μm and a dielectric constant of 3) across the samples for prolonged periods without electrical breakdown. It is important to stress that all electroabsorption experiments were performed on dry samples which passed a negligible electrical current ($\leq$5 nA at 4000 V). Thus, electrochemical processes played no role in these measurements.

Differential absorption spectra were measured after applying a voltage across the sample, using the spectrum recorded at zero voltage as a reference. We used both dc voltages, produced by a high-voltage power supply, and pulses with rise times as fast as 3 msec, generated by using a pulse generator and a wideband high-voltage amplifier. The polarity was reversed after each measurement to avoid any cumulative polarization of the samples. A millisecond scanning spectrophotometer [OLIS (Bogart, GA) RSM-1000] was used to follow the absorption kinetics; we also used a conventional photodiode-array spectrophotometer for some measurements.

RESULTS AND DISCUSSION

Fig. 1 shows electroabsorption spectra for a dry oriented film of wild-type BR. The dashed curve, measured with a negative potential applied to the cytoplasmic side of the film, exhibits a decrease in the ground-state absorption centered at 530 nm and a narrower positive peak at 620 nm. In agreement with previous observations, this difference spectrum resembles that of the acid-blue form of BR and is probably associated with electric-field-induced protonation of residue Asp-85 (7, 8). The full curve was obtained with the cytoplasmic side of the sample held positive. Fields with this polarity produce considerably less electroabsorption; the deviations from zero absorption change can be attributed in part to imperfect orientation and in part to scattering by the Mylar insulator, which wrinkles slightly under the applied field because of electrostriction. Unoriented films exhibit an electroabsorption spectrum which is approximately equal to the average of the two curves in Fig. 1, independent of polarity. Thus, the electroabsorption effect in wild-type BR is completely vectorial.

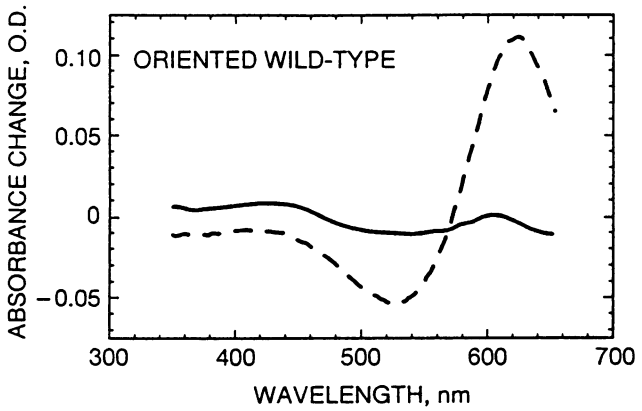

FIG. 1. Electroabsorption spectra recorded for an oriented wild-type BR film of peak absorbance A_{560} = 1.2, equilibrated at 12% relative humidity, at applied potentials of ±4000 V. Dashed line, cytoplasmic side negative; solid line, cytoplasmic side positive. In this and subsequent spectra, noise has been reduced by using smoothed, parabolic B-spline fits.

Fig. 2 shows analogous results for films of D85N BR, obtained under similar conditions. The solid line in Fig. 2 *Upper* shows the electroabsorption spectrum for an electrophoretically sedimented film with the cytoplasmic side held positive. In contrast to the results for wild-type films, this electroabsorption spectrum exhibits a dip in the ground-state absorption centered at 600 nm and an increase in the absorption band centered at 410 nm. Reversing the field polarity (dashed line in Fig. 2) eliminates most of the effect, suggesting that the film is strongly but not completely oriented. In unoriented D85N films (Fig. 2 *Lower*), the electroabsorption spectra for both field polarities are approximately equal to the average of the two spectra in Fig. 2 *Upper*. As in wild-type BR, the electroabsorption effect in D85N BR is vectorial. The voltage dependence of the amplitude of the electroabsorption spectrum in oriented D85N films is quadratic, with a threshold (Fig. 3), as seen in previous measurements on oriented and unoriented wild-type films (7, 8). At the highest applied voltages, the fractional absorption change at 600 nm is $\approx$25% and can be seen by eye.

The kinetic behavior of the absorbance at 410 nm and at 600 nm triggered by the application of a 1-sec positive voltage pulse to the cytoplasmic side of an oriented D85N film is shown in Fig. 4. A rapid change in the applied field triggers a fast transition in the absorption spectrum (time scale < 0.1 sec) followed by a slower component (time scale 1–10 sec). Over half of the total absorption changes at both wavelengths occur on the 30-msec time scale of the applied voltage transitions. In complementary experiments on unoriented D85N films using voltage pulses with faster transition times, we found that 20–30% of the total field-on absorption change occurred within the 3-msec transition time of the applied voltage. Longer kinetic records reveal that the absorption spectrum returns to the baseline after the electric field is switched off,

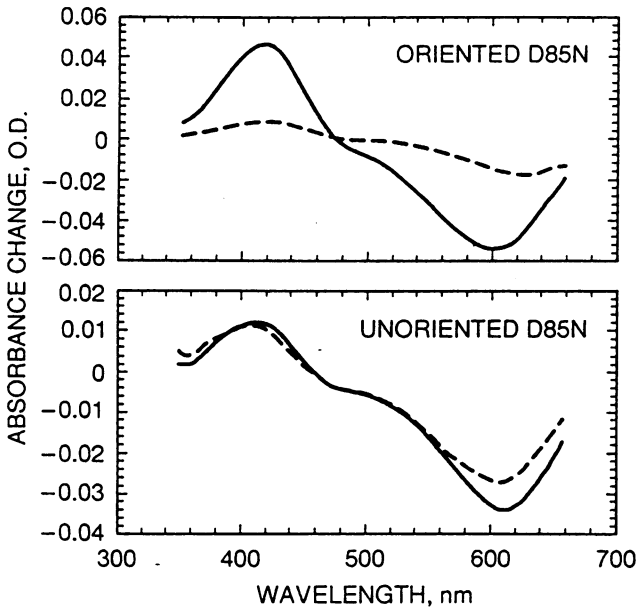

FIG. 2. Electroabsorption spectra of D85N BR films dried over P_2O_5. These data were acquired by applying 3800 V across the sample, waiting 3 min for transients to decay (see Fig. 4), and then averaging the absorption spectrum for 1 min. (*Upper*) Electrophoretically sedimented film; peak absorbance A_{600} = 0.4. Solid line, cytoplasmic side positive; dashed line, cytoplasmic side negative. The vectorial nature of these spectra indicates that this sample was well oriented. (*Lower*) Unoriented film; peak absorbance A_{600} = 0.5. Solid line, substrate positive; dashed line, substrate negative.

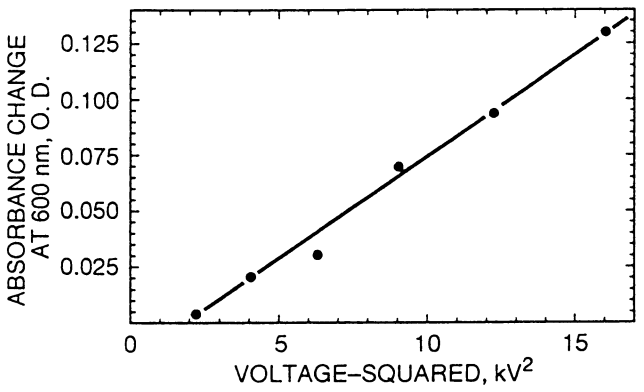

FIG. 3. The amplitude of the electroabsorption spectrum for an oriented D85N film of peak absorbance A_{600} = 0.6, dried over P_2O_5, is plotted as a function of the square of the applied voltage. In this series of measurements, the cytoplasmic side of the film was held positive. The dependence on applied voltage is seen to be quadratic, with a threshold.

even for very long voltage pulses. This indicates that the spectral changes induced by the field pulse are reversible.

The origin of the electric-field-induced spectral changes reported in this paper is made clear by the measurements of Fig. 5, which show the effect of chemical deprotonation of the Schiff base in an oriented D85N film at room humidity. The absorption spectrum of the film as deposited from a suspension at pH 6.0 (Fig. 5 *Upper*, right-hand peak) exhibits a peak centered at 600 nm. The red shift of this peak from its position at 570 nm in wild-type BR is due to the elimination of the negatively charged Asp-85 residue (10). Soaking the film for 30 min in a solution at pH 11.8 causes a shift of this absorption peak to 400 nm, due to deprotonation of the Schiff base (left-hand peak). The difference between these two spectra is shown as the solid line in Fig. 5 *Lower*. This difference spectrum is the signature of Schiff-base deprotonation. The

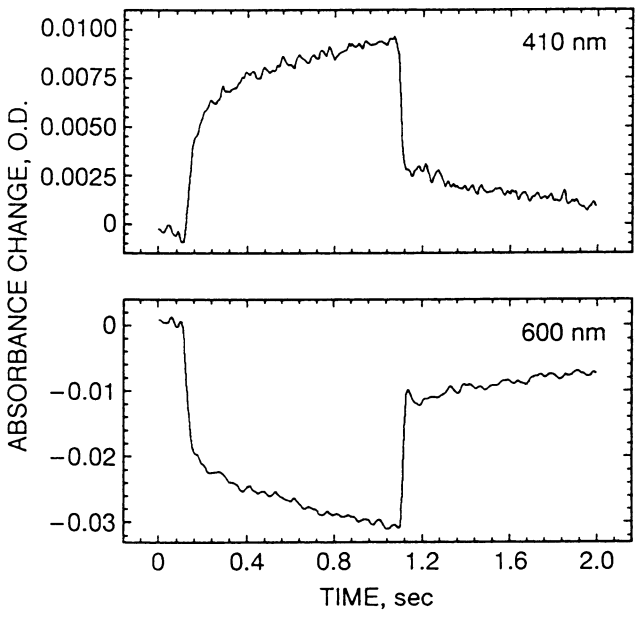

FIG. 4. Kinetic response of a dry oriented D85N film with peak absorbance A_{600} = 0.4 to a 1-sec voltage pulse with amplitude 3500 V and rise and fall times 30 msec. (*Upper*) Absorbance change at 410 nm as a function of time. (*Lower*) Absorbance change at 600 nm. Despite the application of optimized Wiener filtering (15) for noise reduction, which smooths out the fast transitions in these traces, it can be seen that the field-on transition at 0.12 sec is slower than the field-off transition at 1.12 sec.

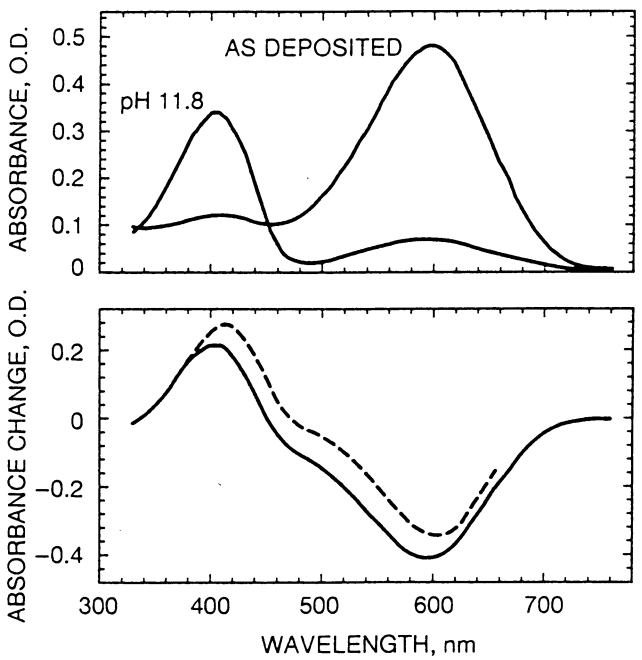

FIG. 5. (*Upper*) Optical absorption spectra of an oriented film of D85N BR at room humidity, after equilibration with solutions at different pH values. The film as deposited at pH 6.0 exhibits a strong absorption centered at 600 nm (right-hand peak). After soaking in an NaOH solution at pH 11.8, this absorption shifts to 400 nm (left-hand peak) due to deprotonation of the Schiff base. (*Lower*) Solid line, difference between these two spectra. This difference-spectrum shape is the unambiguous signature of Schiff-base deprotonation. Dashed line, average of the electric-field-induced difference spectra shown in Fig. 2 *Upper*, magnified by ×10.

dashed line in Fig. 5 *Lower* shows the effect of electric fields on an oriented dry D85N film: this curve is the average of the two electroabsorption spectra shown in Fig. 2 *Upper*, multiplied by a factor of 10. Aside from a slight red shift (possibly a humidity effect) and a baseline shift (possibly an electrostriction effect, as noted above), these two curves are nearly identical in shape. The meaning of this similarity is clear: the application of an electric field to a D85N film produces deprotonation of the Schiff base in a fraction of the protein molecules. This interpretation is consistent with the results of resonance Raman-scattering experiments at λ_{ex} = 406.7 nm. Raman spectra of blue D85N films exhibit a very weak peak at 1562 cm^{-1}. This peak is greatly enhanced by exposure to an alkaline solution, consistent with the previous observation that the appearance of the retinal C=C stretch mode at 1562 cm^{-1} is associated with the shift of the visible absorption peak to 400 nm in the deprotonated state (16). Applying an electric field across a blue D85N film also produces a strong increase in the intensity of the 1562-cm^{-1} peak.

The opposite effect to that just described, namely, the electric-field-induced protonation of the Schiff base in D85N films soaked in alkaline solutions, has also been observed. After equilibration at pH 11.0, the electroabsorption spectrum produced by applying +3800 V to the cytoplasmic side of an oriented film exhibits a shift from 400 nm to 600 nm. The magnitude of the spectral shift after equilibration at this pH is weaker than that seen in the solid curve in Fig. 2 *Upper* by a factor of ≈4; with polarity reversed, the electroabsorption magnitude is below the noise level. When the pH is increased to 11.8, the magnitude becomes too weak to be detected.

The reversible nature of the observed Schiff-base deprotonation and the fact that it is observed in dry films which pass little electrical current indicate that the effect is intrinsic and not due to electrochemistry. One possible artifactual cause of

this effect could be proton transport under the electric field, which would produce a local pH increase, and thus Schiff-base deprotonation, on one side of the film only (17). However, this mechanism would not be eliminated by reversing the field polarity; thus, the vectorial nature of the electroabsorption spectra in Fig. 2 eliminates this possibility. The fast kinetics observed in Fig. 4 also point toward proton motion inside the membrane channel as the source of the observed electroabsorption spectra. Stopped-flow measurements of the spectral changes caused by sudden changes of pH in a suspension of D85N membranes revealed a time constant for Schiff-base deprotonation of 50 sec, presumably determined by diffusion of protons out of the membrane channel and into the surrounding solution (18).

The observations reported in this paper can be understood empirically. Even in dry BR films, the periplasmic and cytoplasmic halves of the proton channel contain small numbers of water molecules (19, 20). External electric fields can in principle drive fast proton transport through either of these hydrogen-bonded water networks. The periplasmic half of the channel of D85N BR contains the negatively charged residue Asp-212, which makes up part of the complex counterion to the Schiff base. A positive voltage applied to the cytoplasmic side of the protein should increase the electrostatic field at the Schiff base due to this negative charge, enhancing deprotonation. There is no corresponding negatively charged residue in the cytoplasmic side of the proton channel; thus, a field of the opposite polarity does not cause deprotonation. When the Schiff base is chemically deprotonated by equilibration at high pH, applying a positive potential to the cytoplasmic side of the protein can in principle reprotonate the Schiff base from the protonated residue Asp-96 in the cytoplasmic side of the channel. The pK of this residue is approximately 10; thus, equilibrating the film at a pH much higher than this should abolish this mechanism, as we observe at pH 11.8. Of course, this picture is only a conjecture. Clearly, experiments at different pH values with the D85N/D96N, D85N/D212N, and D85N/D96N/D212N mutants of BR, in which the charge states of these crucial residues are altered, will yield insight into the nature of the electrostatic forces on the Schiff-base proton. These electrostatic effects could be enhanced by distortion of the protein by electrostatic forces exerted by the external field on charged groups in the proton channels—specifically, Asp-212. Clarification of the relative importance of externally imposed electrostatic forces and the effects of distortion of the protein will also be important in understanding our results. This will require application of the numerical techniques of recent calculations of the electrostatic structure of wild-type BR (13, 14). Our results may require the imposition of new constraints on the structure of the protein. Any model applied to this system will have to account for the quadratic dependence of the Schiff-base deprotonation on the electric field.

Our observations are potentially of great technological importance. The electric-field-induced deprotonation of the Schiff base we observe is a novel electrochromic effect: a switching between two widely separated optical absorption peaks rather than just a small spectral shift of a single peak. Once the sensitivity of this effect is improved, through chromophore substitution or additional genetic modification, the resulting "electronic ink" will be suitable for use in reflective flat-panel displays.

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1. Ebrey, T. G. (1993) in *Thermodynamics of Membrane Receptors and Channels*, ed. Jackson, M. B. (CRC, Boca Raton, FL), pp. 353–387.
2. Lanyi, J. K. (1993) *Biochim. Biophys. Acta* **1183,** 241–261.
3. Becher, B. & Cassim, J. Y. (1975) *Prep. Biochem.* **5,** 161–178.
4. Váró, G. (1981) *Acta Biol. Acad. Sci. Hung.* **32,** 301–310.
5. Oesterhelt, D., Bräuchle, C. & Hampp, N. (1991) *Quart. Rev. Biophys.* **24,** 425–478.
6. Birge, R. R. (1990) *Annu. Rev. Phys. Chem.* **41,** 683–733.
7. Kononenko, A. A., Lukashev, E. P., Maximychev, A. V., Chamorovsky, S. K., Rubin, A. B., Timashev, S. F. & Chekulaeva, L. N. (1986) *Biochim. Biophys. Acta* **850,** 162–169.
8. Tsuji, K. & Hess, B. (1986) *Eur. Biophys. J.* **13,** 273–280.
9. Sheves, M., Albeck, A., Friedman, N. & Ottolenghi, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3262–3266.
10. Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G. & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1018–1022.
11. Marti, T., Roesselet, S. J., Otto, H., Heyn, M. P. & Khorana, H. G. (1991) *J. Biol. Chem.* **266,** 18674–18683.
12. Rousso, I., Friedman, N., Sheves, M. & Ottolenghi, M. (1995) *Biochemistry* **34,** 12059–12065.
13. Sampogna, R. V. & Honig, B. (1994) *Biophys. J.* **66,** 1341–1352.
14. Scharnagl, C., Hettenkofer, J. & Fischer, S. F. (1995) *J. Phys. Chem.* **99,** 7787–7800.
15. Press, W. H., Teukolsky, S. A., Vetterling, W. T. & Flannery, B. P. (1992) *Numerical Recipes* (Cambridge Univ. Press, Cambridge, U.K.), 2nd Ed., pp. 539–542.
16. Nilsson, A., Rath, P., Olejnik, J., Coleman, M. & Rothschild, K. J. (1995) *J. Biol. Chem.* **270,** 29746–29751.
17. Liu, S. Y. & Ebrey, T. G. (1987) *Photochem. Photobiol.* **46,** 557–559.
18. Kataoka, M., Kamikubo, H., Tokunaga, F., Brown, L. S., Yamazaki, Y., Maeda, A., Sheves, M., Needleman, R. & Lanyi, J. K. (1994) *J. Mol. Biol.* **243,** 621–638.
19. Papadopoulos, G., Dencher, N. A., Zaccai, G. & Büldt, G. (1990) *J. Mol. Biol.* **214,** 15–19.
20. Fischer, W. B., Sonar, S., Marti, T., Khorana, H. G. & Rothschild, K. J. (1994) *Biochemistry* **43,** 12757–12762.